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FROM YOUTH TO ADULT: STUDIES ON CHRONIC AIRWAY OBSTRUCTION WITH SPECIAL REFERENCE TO EVENTS IN THE NEONATAL PERIOD

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FROM YOUTH TO ADULT: STUDIES ON CHRONIC AIRWAY OBSTRUCTION WITH SPECIAL REFERENCE TO EVENTS IN THE NEONATAL PERIOD

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To all my patients, without you I would not have written this thesis

and

To my father, who once said: just don’t stop studying…

ABSTRACT

Introduction: In western countries, 6-12% of all pregnancies end preterm, i.e. before 37 weeks of gestation. After the introduction of antenatal corticosteroids and improvements of neonatal intensive care, survival rates for children born preterm have increased worldwide. In Sweden, 80% of infants born extremely preterm, i.e. before 28 weeks of gestation, survive. There is, however, an association between preterm birth and later sequelae. Pulmonary complications, such as bronchopulmonary dysplasia (BPD), may result in lung function impairment later in life. It has also been demonstrated that a significant proportion, around 20-25%, of patients with chronic obstructive pulmonary disease (COPD) have never smoked, suggesting other risk factors for developing chronic airway obstruction in adulthood. As there are now more survivors reaching adult life, individuals born preterm are expected to be an increasing group of patients in the future, contributing to the non-smoking proportion of patients with COPD. The overall aim of this thesis was therefore to extensively characterise clinical, functional and mechanistic aspects of pulmonary outcomes in adolescence and in adult age in individuals born preterm with and without BPD.

Patients and methods: Individuals born before 32 weeks of gestation between 1992 and 1998 in Stockholm County were investigated both in adolescence (n= 51) and at adult age (n= 49). Half of the individuals born preterm had a diagnosis of BPD. In adult age two control groups of patients with allergic asthma (n= 23) and healthy individuals (n=24) were included. Information on perinatal data, medical history and health related quality of life (HRQoL) was collected. Lung function was measured using dynamic spirometry, body plethysmography diffusing capacity for carbon monoxide (DL_{CO}), impulse oscillometry (IOS) and lung clearance index (LCI) for ventilation inhomogeneity. Bronchoscopy was performed in adults (mean age 20.0 years) including sampling of the large (bronchial wash), and small airways (bronchoalveolar lavage, BAL).

Results: Both adolescents and adults with BPD demonstrated airway obstruction in contrast to individuals born preterm without BPD, but both groups had more airway hyper-responsiveness compared to healthy controls. In adulthood, the preterm group had lower DL_{CO} irrespective of BPD status, but only those with BPD had signs of inhomogeneous ventilation. Adults with BPD reported fewer physical symptoms than asthmatic controls, despite lower lung function in the former group. Both preterm groups reported lower scores in the mental component summary of a questionnaire compared to healthy controls. In contrast to the asthmatic group, no eosinophilic inflammation was seen in the preterm group. In BAL, the preterm BPD group showed an increased proportion of activated cytotoxic T cells ($CD8^+$), a decreased proportion of T helper cells ($CD4^+$), and, as a consequence, a decreased CD4/CD8 ratio, when compared to the healthy controls. T-cell subsets in BAL correlated with measures of airflow limitation in individuals with BPD. Further, in bronchial wash, elevated proportion of lymphocytes was observed. A correlation of lymphocyte count with measures of airflow obstruction in the preterm born individuals was seen predominantly in males.

Conclusions: Individuals born preterm with a history of BPD have obstructive airflow limitations engaging the small airways. Lymphocytes may have a sex-specific role, as an increased amount was found in the large airways in males with BPD. The increased proportion of cytotoxic ($CD8^+$) T cells in BAL resemble features of COPD, and are compatible with the hypothesis that T-cells may play a mechanistic role in development of airway obstruction in adults with a history of BPD.

LIST OF SCIENTIFIC PAPERS

I. **Lung function development after preterm birth in relation to severity of Bronchopulmonary Dysplasia.**
Um-Bergström P, Hallberg J, Thunqvist P, Berggren-Broström E, Anderson M, Adenfelt G, Lilja G, Ferrara G, Sköld CM, Melén E. *BMC Pulmonary medicine*, 2017, 17, 97

II. **Pulmonary outcomes in adults with a history of Bronchopulmonary Dysplasia differ from patients with asthma.**
Um-Bergström P, Hallberg J, Pourbazargan M, Berggren-Broström E, Ferrara G, Eriksson MJ, Nyrén S, Gao J, Lilja G, Lindén A, Wheelock ÅM, Melén E, Sköld CM. *Respiratory Research* 2019 May 24;20(1):102

III. **Increased $CD8^+$ T-cells in bronchoalveolar lavage (BAL) fluid in adults with a history of bronchopulmonary dysplasia.**
Um-Bergström P, Pourbazargan M, Levänen B, Ström M, Gao J, Berggren-Broström E, Melén E, Wheelock ÅM, Lindén A, Sköld CM.
Manuscript

IV. **Elevated lymphocytes in the large airway in adults born prematurely with a history of bronchopulmonary dysplasia**.
Gao J, **Um-Bergström P**, Pourbazargan M, Berggren-Broström E, Li CX, Merikallio H, Kaarteenaho R, Reinke S, Wheelock CE, Melén E, Rassidakis G, Lindén A, Wheelock ÅM, Ortiz-Villalon C, Sköld CM.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADD	Attention deficit disorder
ADHD	Attention deficit/ hyperactivity disorder
APC	Antigen presenting cells
ATS	American Thoracic Society
AX	Area of reactance
BPD	Bronchopulmonary dysplasia
BW	Birth weight
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonry disease
CRP	C-reactive protein
DCD	Developmental coordination disorder
DL	Diffusing capacity in the lungs
DL_{CO}	Diffusing capacity in the lungs of carbon monoxide
ERS	European Respiratory Society
ERV	Expiratory Reserve Volume
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory flow volume in 1 second
FOXP3	Forkhead box P3
FRC	Functional residual capacity
FVC	Forced vital capacity
GA	Gestational age
GINA	Global Initiative for Asthma
GLI	Global Lung Initiative
GOLD	Global Initiative for chronic Obstructive Lung disease
HLA	Human leukocyte antigen
IC	Inspiratory capacity
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IOS	Impulse oscillometry

IRV	Inspiratory reserve volume
IUGR	Intrauterine growth restriction
LCI	Lung clearance index
LLN	Lower limit of normal
LUNAPRE	LUNg obstruction in Adulthood of PREmaturely born
MBW	Multiple breath washout
MCS	Mental component summary scores
MHC	Major histocompability complex
nCPAP	Nasal continuous positive airway pressure
NK	Natural killer cells
NK-T cells	Natural killer T cells
PALM	Psychology, Allergy, Lung function and Motor development
PC	Provocative concentration
PCS	Physical component summary scores
PD	Provocative dose
PDA	Patent ductus arteriosus
PEF	Peak expiratory flow
PPV	Positive-pressure ventilation
R5-20	Frequency dependence of resistance
RDS	Respiratory distress syndrome
ROP	Retinopathy of prematurity
RV	Residual volume
SF36	Short form health survey -36
SGA	Small for gestational age
SGRQ	St George's Respiratory Questionnaire
SHS	Second hand smoking
TCR	T-cell receptors
Th cells	T- helper cells
TLC	Total lung capacity
TNFα	Tumour necrosis factor alpha
Treg	Rregulatory T Cells
T_V	Tidal volume
VC	Vital capacity

1 INTRODUCTION

In western countries, approximately 6 to 12 per cent of all pregnancies end preterm (before 37 weeks of gestation).[1,2] After the introduction of antenatal corticosteroids 35 years ago and improvement of neonatal intensive care, survival rates after preterm birth have increased worldwide.[3] In Sweden 80% of infants born extremely preterm (before 28 weeks of gestation) survive.[4] There is an association between preterm birth and later complications during the neonatal period, during childhood and into adult life. For instance, preterm birth is associated with development of obstructive lung disease, cardiovascular diseases, diabetes mellitus and cognitive disorders. Individuals with airway obstruction associated with preterm birth are expected to be a growing group of patients in the future since there are now more survivors reaching adult life.[5-7] It is difficult to identify which children born preterm who are at risk for developing chronic lung disease in adulthood as there currently are no known biomarkers to evaluate lung damage due to preterm birth. Children born preterm with respiratory problems are treated with bronchodilators, inhaled corticosteroids and leukotriene receptor antagonists even though this is not evidence based medicine.[8,9] Very little is known if there is an ongoing inflammation or presence of structural changes in the lungs and how this is related to functional parameters or clinical symptoms. It is therefore important to characterise both clinic/symptoms and molecular changes in the lung in children born preterm in order to better understand the connection between perinatal factors and chronic lung disease in adulthood.

2 BACKGROUND

2.1 THE RESPIRATORY SYSTEM

The respiratory system includes the airway tree, the lung parenchyma and pulmonary vessels. The main function is to provide oxygen to the body and to remove carbon dioxide from the bloodstream. The airway tree has approximately 23 generations of branches which are divided into conducting airways generation (the first 15 generations), and intra-acinar airways. In the conducting airways there is no gas exchange and the lining consists of bronchial epithelial cells. The term “small airways” refers to airways from generation eight and more peripheral and with a diameter of 2 mm or less in adults. Gas exchange occurs from the intra-acinar airways where the alveoli begin to appear. The alveoli have a thin one cell layer wall and are surrounded by a network of capillaries allowing exchange of oxygen and carbon dioxide. In the last generation alveoli forms blind ending alveolar sacs.[10]

2.2 THE NORMAL LUNG DEVELOPMENT

The development of the normal lung is a process that begins in the embryonic phase and continues after birth into childhood.[11,12] Furthermore, there are new data suggesting that lung development is an even longer ongoing process proceeding into adolescence and early adulthood.[13]

The **embryonic phase** is the first of the five phases of lung development and occurs during week 4-7 of gestation. This phase begins with the formation of a groove in the ventral lower pharynx, sulcus laryngotrachealis. Within a couple of days buds form the lower parts, the lung primordia. The development continues with further subdivision into the two main bronchi where the left one is directed more laterally than the larger one on the right side, which is directed more caudally. The subsequent lung growth continues to be asymmetric with formation of three endodermal buds on the right side and two on the left side. These buds will further divide into the segments of the individual pulmonary lobes at the end of the embryonic phase. The vasculogenesis occurs within the immature mesenchyme.
The next phase that occurs is the **pseudoglandular phase** between gestational weeks 5-17. All the phases are somewhat overlapping in the beginning and at the end of each phase. In this phase the lung resembles the development of a tubule-acinus gland. The bronchial buds are now undergoing a rapid dichotomous branching and the entire air-conducting bronchial tree up to the terminal bronchiole (i.e. the first16 generations) is formed. At the end of this phase there are up to 20 generations present in some parts of the lung. These lower generations represent respiratory ducts. At the 10th week of gestation cartilage, smooth muscle cells and bronchial glands can be found in the bronchial walls. The cells coating the air-conducting bronchial tree are initially cubic epithelia which are the precursor cells of the ciliated epithelium and of the secretory cells. The first ciliated epithelial cells occur in the 13th week of gestation. Type II pneumocytes appears in the respiratory part of the terminal bronchiole. The epithelium of the maturing lung then begins to produce lung fluid.[11]
During the **canalicular phase** (weeks 16-27), canaliculi expand out of the terminal bronchiole. They represent the proper respiratory part, the pulmonary parenchyma. At the terminal bronchioles the acini are formed with respiratory bronchiole, the alveolar ducts and later the alveolar sacculi. Along the acinus a massive migration of capillaries into the mesenchyme occurs. The flattened type I pneumocytes develop from the cubic type II pneumocytes. This latter step, with differentiation of sufficient levels of type II pneumocytes to type I pneumocytes and proliferation of capillaries is crucial for survival of infants born preterm. Synthesis and storage of surfactant can be seen in the type II

pneumocytes. In the **saccular phase** from gestation weeks 24-38 cluster of sacs develop on the terminal bronchiole. They are the last generation of conducting airways and also the terminal respiratory part of the bronchial tree. In the succuli type I and II pneumocytes cover the surface. The type II pneumocytes start to secrete surfactant into the foetal lung fluid. The primary septa between sacculi are thick and contain a double capillary network. The interstitial space contains a great amount of cells compared to the proportion of collagen and elastic fibres. Interstitial fibroblast starts to produce extracellular material at the end of this phase. The **alveolar phase** is the last phase of lung development. The first part of this phase takes place from gestational week 36 to three years of age, when the alveoli are formed by secondary septa that divide the succuli into thin walled structures with a capillary bed. The thin layers enhance the gas exchange between the air and the blood. The one layer epithelium of the alveoli consists of type I and type II pneumocytes. Recent studies suggests that the process is continued with a second phase, with a refining of the capillary bed between the alveoli to a single layer of capillaries continuing into early adulthood.[12]

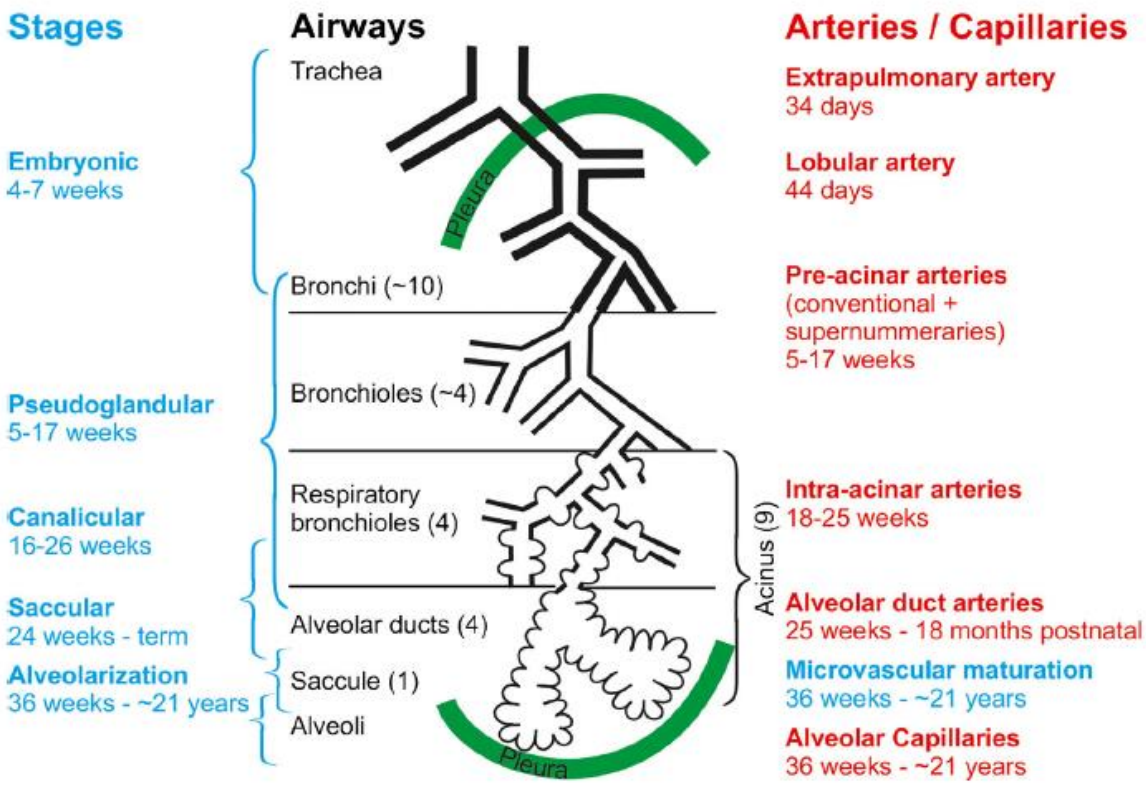

Figure 1. Development of the lung (blue), the airways (black), and the arteries (red). Reproduced from Schittny JC.[12] With permission from Springer; Cell and Tissue Research, 2017.

2.3 THE IMMUNE SYSTEM

2.3.1 The innate immune system

The innate immune system of the airways and the lung consists of cells and mediators serving as the first line defence against invasion of different pathogens. It can act fast, immediate or within hours, when recognizing a potential pathogen. The structural cells involved to produce a barrier to infections are ciliated cells, mucus-secreting goblet cells, club cells producing antimicrobial compounds, and basal cells.[14] Innate immune cells in the tissue are including macrophages, dendritic cells, neutrophils, eosinophils, monocytes, innate lymphoid cells (ILC), natural killer cells (NK), and mast cells (Figure 2).[15]

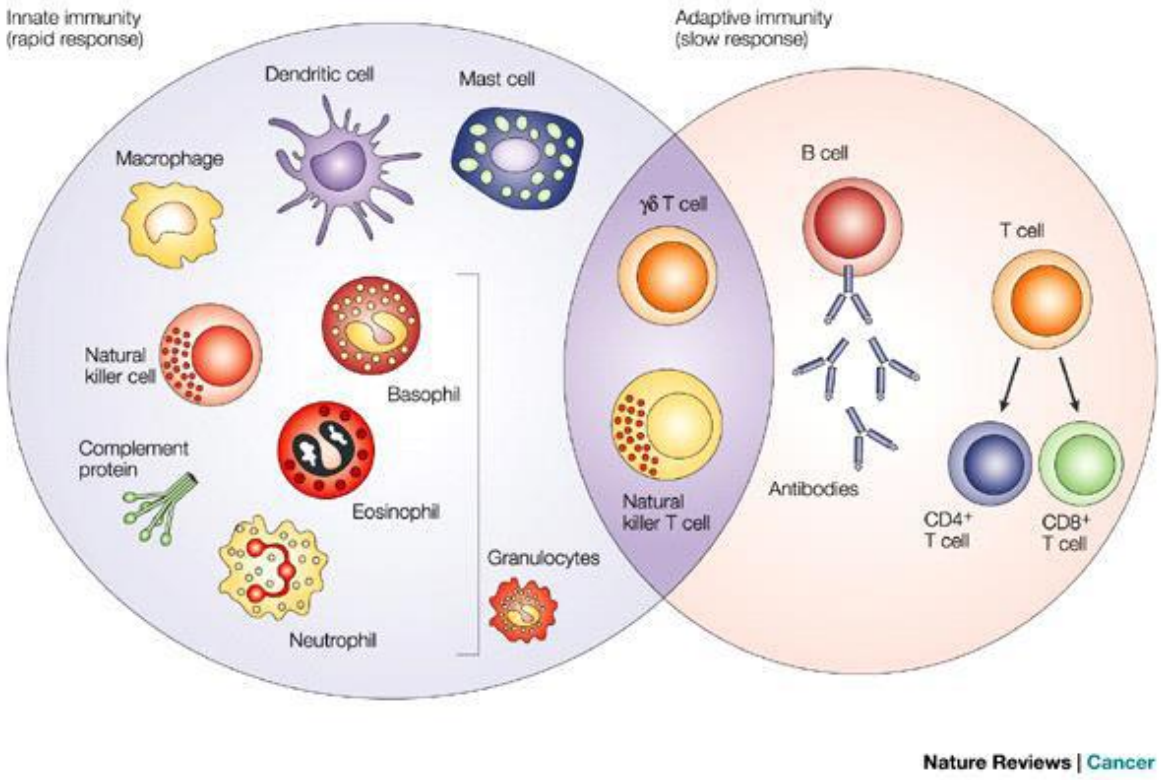

Figure 2. Cells involved in the innate and adaptive immune system.
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2.3.2 The adaptive immune system

The adaptive immune response is antigen specific but slower, and has evolved to recognize both self- and nonself- antigens. It consists of lymphocytes and their products (Figure 2). Adaptive immune response depends on receptors that are tailored to be specific. The receptors are selected through a process of recombination of different gene segments. The

adaptive immune system contains immunologic memory which gives a more effective response to multiple exposures of the same antigen.[16]

2.3.2.1 *T cells*

Progenitor T cells originate from the bone marrow and migrate to the thymus where they mature. T cells express specific antigen-binding receptors on their surface called T-cell receptors (TCR). T cells demand action from antigen presenting cells (APC) such as dendritic cells, macrophages, B cells, fibroblasts, and epithelial cells to be able to recognize specific antigens. When receiving an appropriate signal the T cell can proliferate and differentiate rapidly. Bound to the surface of APC there are proteins called major histocompability complex (MHC). These are either MHC class I (in humans also called human leukocyte antigen [HLA] A, B and C) which are expressed on all nucleated cells, or MHC class II (also called HLA DP, DQ and DR) which are only expressed by some cells in the immune system for instance macrophages, dendritic cells and B cells. The cells expressing MHC class I mainly present peptides from viruses and aberrant cells, thereby enabling the activation of cytotoxic T cells ($CD8^+$ cells). Activation of CD^+8 cells is followed by clonal expansion which leads to production of effector cells. Effector cells activate apoptotic cell death within minutes after binding to complex of peptides bound to MCH class I on the target cell. This process involves exocytosis of granules including granzymes and perforins. Another mechanism of apoptosis is driven by expansion of the Fas ligand on the $CD8^+$ cells. Apoptosis of the target cell is triggered when this ligand binds to Fas (CD95).[17] The cells expressing MHC class II internalize and degrade extracellular pathogens for example bacteria and then display peptides to T- helper (Th) cells ($CD4^+$ cells). $CD4^+$cells are unable to directly kill infected cells or clear pathogens. Instead they work by releasing cytokines and further conduct other cell types, including the APCs to perform these tasks. The activation of $CD4^+$ cells lead to differentiation into several types of effector cells of which Th1, Th2 and Th17 subsets are the most common. The characteristics of Th1 response is secretion of interferon (IFN)-γ and interleukin (IL)-2 targeting macrophages to act in defence against intracellular pathogens. Typical for the Th2 response is secretion of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 which target eosinophils in the defence against parasites (helminths) whereas the Th17 produces IL-17 and IL-22 to engage neutrophils to work against extracellular pathogens. There is a subset of $CD4^+$ T cells called regulatory T Cells (Treg) that possesses immunoregulatory functions to suppress inflammation and inhibit autoimmunity. The Tregs express a transcription factor called forkhead box P3 (FOXP3) and a bright expression of the surface marker CD25.[18,19]

The γδ T cells are abundant in epithelia in the gut but represent only around 5% of all T cells. They neither express CD4 nor CD8.[17] Natural killer T cells (NK-T cells) are a small population, less than 5% of all T cells, which express both αβTCR and the same markers as NK cells such as CD56 and CD16.[20] Activated NK-T cells can fast produce high amounts of cytokines.[17]

2.3.2.2 *B cells*

The B cells derive from hematopoietic stems cells in the bone marrow where they mature and develop the ability to generate an antigen specific humoral immune response. The response involves production of antibodies that can neutralize toxins, opsonization of pathogens promoting phagocytosis, complement activation, and cell mediated cytotoxicity. Proteins, lipids, carbohydrates, macromolecules and smaller chemicals can be recognized by B cells directly, without the need for APCs, by their unique cell surface bound antibodies. When activated by an antigen the B cells proliferate and differentiate into plasma cells secreting antibodies, or into memory B cells. The memory B cells are long-lived and have a rapid response when coming across the same antigen again resulting in fast production of antibodies and elimination of the targets. The plasma cells undergo apoptosis when the targets are eliminated. Thus, they are short lived; they are very effective as they produce copious amounts of antibodies.[16] B cells produce five major types of antibodies: Immunoglobulin (Ig)A, IgD, IgE, IgG and IgM. IgG can be further sub-classified. The different classes of antibodies have contrasting functions and distinguish diverse pathogens. B cells also act by cell-mediated immunity without any involvement of antibodies but through activation of antigen-specific cytotoxic T cells, macrophages, NK cells and thereby stimulation of cytokine production. The cell mediated immunity is directed to effective elimination of virus-infected cells and cancer cells but also has a role in the defence against intracellular bacteria, protozoes, and fungus.

2.3.3 Microbiota in the airways

Development of the microbiota after birth has an impact on the immune system and future health. It is influenced by many factors, such as mode of delivery, breast feeding, genetics, environmental factors, and treatment with antibiotics.[21,22] The microbiota of the airways serves as protection against colonization by pathogens. The respiratory microbiota might also play a role in the homeostasis of respiratory physiology and immunity.[23] However, the precise role of the microbiota remains to be investigated.

2.4 FACTORS AFFECTING LUNG DEVELOPMENT

2.4.1 Maternal/paternal factors

Maternal smoking during pregnancy is associated with poor lung function at birth, during childhood and in early adulthood.[24] Infants born preterm exposed to maternal smoking *in utero* have an increased risk of developing BPD.[25,26] In experimental animal models the effects of nicotine on lung development have been demonstrated to cause thicker alveolar walls, increased airway smooth muscle and collagen deposition, and airway hyper responsiveness with airflow restriction.[27,28] Other risk factors for BPD include genetics and hereditary factors. In a large population-based cohort Gage *et al*. found that children born preterm to asthmatic mothers who did not receive antenatal steroids had an increased risk of developing BPD.[29] Influence of genetics has been shown by Bhandari et al in a twin study where they compared the risk of developing BPD, necrotizing enterocolitis and intraventricular haemorrhage in pairs of mono- and dizygotic twins born ≤ 32 weeks of gestation. They found that genetic factors explained 53% of the risk to develop BPD, after adjusting for covariates.[30] Lavoie *et al*. evaluated mono- and dizygotic twins born ≤ 30 weeks of gestation in Canada and demonstrated a substantial genetic contribution to moderate-to-severe BPD.[31] Research has also been focused to identify associations with BPD involving molecules or pathways connected to lung development, inflammation, fibrosis angiogenesis oxidative stress or tissue injury and repair.[32-34] However, many of the associated single nucleotide polymorphisms in candidate genes have not been confirmed in genome-wide association studies on patients with BPD.[35,36]

2.4.2 Pre-/peri-/post-natal factors

2.4.2.1 *Preterm birth*

Preterm birth is defined as delivery before 37 weeks of gestation. Very preterm infants are born <32 weeks and extremely preterm infants <28 weeks of gestation. According to the Swedish National Board of Health and Welfare statistics, 5-6 % of all pregnancies in Sweden end preterm (<37 weeks), about 1% end very preterm and 0.3-0.4% end extremely preterm.[37] Due to preterm birth, lung maturation is disrupted during the canalicular and saccular/early alveolar phases of normal lung development, a process that is supposed to take place before birth. The level of surfactant is insufficient and the structures of the lungs, i.e. airways, vasculature and interstitium, are immature. Antenatal glucocorticoid steroids given to mothers at risk of preterm delivery has improved survival of very preterm infants the last decades. Glucocorticoids accelerate lung maturation in the foetus which reduces the

risk of developing respiratory distress syndrome. It also has the effect of reducing the risk for intraventricular haemorrhage and necrotizing enterocolitis in preterm born infants and reduces mortality in the group.[38,39]

2.4.2.2 *Respiratory distress syndrome (RDS)*

In infants born preterm respiratory distress syndrome (RDS) is a common condition with inadequate gas exchange. Insufficient levels of surfactant in the alveolus causes RDS.[40] The incidence increases with decreasing gestational age (GA). The definition is based on symptoms of respiratory distress (retractions, grunting and tachypnoea), increasing demand for supplemental oxygen and typical chest X-ray findings without any evident other conditions.[40,41] RDS is a common cause for the need of nasal continuous positive airway pressure (NCPAP) or mechanical ventilation. RDS is in modern neonatal care treated by intratracheal administration of exogenous surfactant.

2.4.2.3 *Bronchopulmonary Dysplasia (BPD)*

Some of the infants with RDS at birth will eventually develop bronchopulmonary dysplasia (BPD). Approximately 10-30% of infants born younger than GA 30 weeks and with a birth weight (BW) under 1000 g will develop this condition.[42] Bronchopulmonary dysplasia is often explained as a developmental condition with pathogenesis being linked to immature lungs, inflammation, barotrauma and volutrauma resulting from the use of mechanical ventilators and oxidative stress due to the need for supplemental oxygen.[43] Historically, BPD, now referred to as "old BPD" was associated with inflammation, parenchymal fibrosis and airway injury. However, the achievements in modern neonatal care have been accompanied by a new phenotype with altered disease, where "new" BPD is characterized of even more immature lung tissue affected by reparative processes, impaired alveolarization, and dysmorphic vascular growth.[44-46] BPD is currently defined by the need for supplemental oxygen in 28 days. It can be further classified as moderate or severe BPD based on the level of oxygen need at 36 weeks of gestation (Table 1).[47] To confirm the oxygen requirement at the assessment time point a standardized stepwise oxygen reduction challenge test should be performed. A day of treatment with oxygen > 21% is defined as more than 12 hours with supplemental oxygen within one day. [4,5,42]

Table 1. Definition of Bronchopulmonary Dysplasia according to Jobe and Bancalari[47]

Gestational Age	**< 32 weeks**	**⩾ 32 weeks**
Treatment with oxygen > 21% for at least 28 days **plus**		
Mild BPD	Breathing room air at GA 36 weeks or discharge	Breathing room air by 56 days postnatal age or discharge
Moderate BPD	Need for < 30% oxygen at GA 36 weeks or discharge	Need for < 30% oxygen at 56 days postnatal age or discharge
Severe BPD	Need for ⩾ 30% oxygen and/or positive pressure, at GA 36 weeks or discharge	Need for ⩾ 30% oxygen and/or positive pressure, at 56 days postnatal age or discharge

Abbreviations: BPD = bronchopulmonary dysplasia; GA = gestational age

2.4.2.4 *Chorioamnionitis*

Chorioamnionitis is a risk factor for the development of BPD in more than one way. It is a major risk factor of premature birth, which in turn is the strongest risk factor of developing BPD.[48,49] It also causes chronic inflammation in the lung due to pro-inflammatory cytokines in the amniotic fluid.[50,51] Chorioamnionitis is characterized by an intrauterine inflammation of the choriodecidual space, chorioamniotic membranes, amniotic fluid or the umbilical cord. The clinical findings are maternal fever, elevated white blood cell count, tender uterus, and by amniotic fluid analyses for positive microbial culture or PCR, elevated cytokines (IL-6, tumour necrosis factor α (TNF-α), IL1β and IL-8), or inflammatory cells. The histological signs are granulocyte infiltration and occurrence of necrosis in the choramnion.[52] The organisms found in cultures can be single microbes or polymicrobial of aerobes and anaerobes generally found in vaginal flora. The most common pathogens associated with chorioamnionitis and preterm delivery are *Ureaplasma spp*.[53-55] In contrast to an increased risk of developing BPD, the inflammation driven by chorioamnionitis seems to reduce the incidence of RDS due to earlier lung maturation and induction of surfactant production.[56] It has been difficult to study and establish the consensus of the relationships between chorioamnionitis, RDS and BPD because of the complexity with different antenatal variables and the postnatal exposures with diverse care strategies that contribute to the diagnoses of RDS and BPD. Animal models have provided information on how experimental chorioamnionitis can impact the foetal lung.[57]

2.4.2.5 *Intrauterine growth restriction, small for gestational age*

Five to fifty percent of infants born preterm are growth restricted or small for gestational age (SGA) at birth.[58-60] Intrauterine growth restriction (IUGR) is diagnosed if the foetus does not reach its target weight according to ultrasound estimation for the GA. An estimated weight < 10^{th} percentile is a widely used definition. SGA implies a BW less than two standard deviations (SD) below the mean according to GA. However, IUGR and SGA are not synonymous. IUGR is mirroring a pathological process taken place *in utero*. An infant born SGA has not necessarily suffered from IUGR but could be constitutionally small and an infant with only a short period of IUGR is not always born SGA.[61] According to the foetal origin hypothesis an unfavourable intrauterine environment resulting in IUGR and/or SGA can affect lung development.[62,63] Infants born IUGR/SGA have an increased respiratory morbidity and mortality during the neonatal period.[64,65] IUGR/SGA is associated with decreased lung function in children and adults.[66-69]

2.4.2.6 *Nutrition*

Infants born extremely preterm are at risk for postnatal growth restriction secondary to challenges of delivering optimal nutrition. Despite advances in the use of nutrients for both enteral and parenteral use many preterm infants demonstrate growth failure. Nutrition plays a critical role in the prevention and management of BPD. Malnutrition can worsen BPD by compromising lung growth.[70-72] Murine models of postnatal growth restriction have described pulmonary vascular remodelling, right ventricular hypertrophy, and an altered expression of regulators of lung development including vascular endothelial growth factor.[73] Infants with BPD have been estimated to have a 15% to 25% larger energy need than infants without BPD.[74] Feeding difficulties in these infants can further affect nutrition. Initial parenteral nutrition with protein and lipids, and early enteral feeding may help decrease the incidence of BPD.[75] The use of fortified breast milk and/or formula is necessary to achieve adequate growth if total daily fluid intake is restricted. Nutritional status at 2 years of age is a positive predictor of pulmonary outcomes later in childhood.[76]

2.4.2.7 *Respiratory support*

The practice of mechanical ventilation in preterm infants using adult equipment with adapted devices to fit infants started in the beginning of the 1960s. There were many problems with the use of ventilators in infants born preterm. The ventilators were too rough and they had a large dead space. In addition, they were not able to synchronise with the infants' breathing and it was therefore necessary to sedate and completely paralyse the patients. Before the use of endotracheal tubes all patients were tracheotomised.[77] Despite of

the crude treatment, the preterm born patients had a greater chance to survive than before but many of them developed the old form of BPD.[45]

The first ventilator designed exclusively for paediatric and neonatal use was introduced in 1980. With its introduction infants born preterm could be ventilated on a volume-oriented basis for the first time, as the volume was metered and measured more exact. The use of mechanical ventilation is a well-known risk factor for BPD. It can cause volutrauma when the lungs are inflated with volumes larger than the total lung capacity (TLC), and barotrauma occurs when the lung is over-distended with a disarrangement of structural elements. This will lead to an inflammatory response and an influx of chemokines and cytokines, and a relocation of leukocytes into the lung.[78] Ventilation with volumes below functional residual will lead to collapse and re-expansion of the peripheral airways and alveoli, and causes atelectotrauma due to shear stress and injury.[79] In the last decades, different ventilation strategies to prevent or minimize lung injury have arisen. These are different modes of synchronized ventilation and volume targeted ventilation.[80] The use of noninvasive ventilation strategies such as nasal continuous positive airway pressure (nCPAP) (Figure 3) has been a successful way to reduce the rates of BPD[81-83], and can replace intubation and surfactant as the first line of therapy for many preterm infants.[84,85] High-flow nasal cannula (HFNC) is another form of noninvasive respiratory support that has become more popular and is recently used with increasing frequency, both as primary respiratory support after birth and when weening from mechanical ventilation and nCPAP.[86]

Figure 3.
One-day-old girl with nCPAP, born preterm at 27 weeks gestational age.
With permission from the parents.

2.4.2.8 *Drugs*

Supplemental oxygen

Oxygen therapy is one of the most essential drugs used worldwide in neonatal care. However, exposure to high concentrations of oxygen contributes to the development of BPD. Hyperoxia results in a production of cytotoxic reactive oxygen species which causes an acute pulmonary injury characterized by an inflammatory response with destruction of the alveolar capillary barrier, followed by cell death. This process, with a simultaneous damage and an attempt to repair, mediated via a variety of factors, results in lung pathology that in the end will result in development of BPD in infants born preterm.[87-91] Animal models of oxygen-mediated lung injury have been used in order to address this question.[92,93]

Surfactant

Exogenous surfactant is an established treatment of preterm infants with RDS. It improves gas exchange and survival.[94,95] There are both natural porcine surfactant and synthetic surfactant for commercial use. The use of natural porcine surfactant was developed in 1982 by the Swedish paediatric pathologist Bengt Robertson and clinical chemist Tore Curstedt.[96] The most common way of administration is as a bolus dose through an endotracheal tube that in many cases can be removed soon afterwards (INSURE procedure; INtubation, SURfactant, Extubation).[97,98] Modern minimal invasive approaches have evolved where the infant is breathing spontaneously and the surfactant is instilled by a thin catheter (LISA; less invasive surfactant administration, or MIST; minimal invasive surfactant therapy).[99-101]

Corticosteroids

Due to their anti-inflammatory effects post-natal corticosteroids have been evaluated as both a preventive strategy and potential treatment of BPD. The concerns regarding side effects of systemic use of corticosteroids such as neurodevelopmental impairment, cerebral palsy and death have been confirmed in a recent Cochrane review.[102] Regarding the use of inhaled corticosteroids Shinwell *et al* showed in a meta-analysis a reduction in the number of patients who developed BPD but there were no data available on long term neurodevelopmental outcome or later pulmonary morbidity. Both early prevention and later treatment use of inhaled corticosteroids was included in the analysis.[103] Further research is needed to evaluate the type of inhaled steroid, timing, formulation, dosage, and method of administration that is most appropriate for the prevention and treatment of BPD.[104-106]

Bronchodilators

Salbutamol /Albuterol is an inhaled β2-agonist that is widely used as a bronchodilator for the treatment of BPD. Increased compliance and tidal volumes, as well as decreased pulmonary resistance have been documented in infants with BPD. It has been associated with short-term improvements in pulmonary resistance and lung compliance secondary to bronchial smooth muscle relaxation.[107] A recent Cochrane review examining the role of salbutamol was unable to find sufficient evidence of efficacy in the prevention of BPD. Very few well performed studies were addressing this question.[108] Koch *et al* stated recently that early inhaled bronchodilators did not reduce the risk of BPD and death in extremely preterm born infants.[109] Ipratropium bromide is a muscarinic antagonist resulting in bronchodilation, mainly used for treatment of chronic obstructive pulmonary disease (COPD) in adults. However, the drug is also used in the treatment of infants with BPD, often combined with β2-agonists. Significant improvements in airway resistance and compliance have been shown but long-term effects of pulmonary function have not been demonstrated.[110-112]

Caffeine

Caffeine is the most common used methylxanthine to prevent apnoea of prematurity, but is also routinely used among infants born preterm for prevention of BPD. It has been shown to reduce the time of mechanical ventilation, CPAP and supplemental oxygen and hence the risk of developing BPD.[113,114] The specific mechanism by how caffeine is protective against lung injury remains unclear, but it has been shown to increase respiratory drive, diaphragm contractility, and pulmonary compliance while reducing airway resistance.[115]

Diuretics

To provide preterm infants adequate hydration and nutrition large fluid volumes are often given. Excessive fluids administered can be associated with pulmonary oedema, and in turn an increased need for respiratory support and an increased risk of developing BPD. Despite limited data regarding efficacy, diuretics are commonly used in preterm infants to improve respiratory status. Furosemide is a type of loop diuretics that decreases interstitial oedema and pulmonary vascular resistance. If used after three weeks of age furosemide improves oxygenation and lung compliance. However, long-term benefits have not been established in infants with BPD.[116] Adverse effects with long term use are nephrocalcinosis, and loss of hearing.[117] Thiazide diuretics differ from loop diuretics with respect to mechanism, mode of action, efficacy, and side effects. As with Furosemide it seems to have short term effects on pulmonary mechanics but long term effects on respiratory outcomes are unknown.[118] Spironolactone is a synthetic steroid that acts as a competitive aldosterone receptor

antagonist. Spironolactone is a weak diuretic and is primarily used in the neonatal population for its potassium-sparing effects, typically in combination with a thiazide diuretic.[117]

Inhaled Nitric Oxide

In animal models inhaled nitric oxide (iNO) stimulates pulmonary angiogenesis, and reduces inflammation and lung tissue damage.[119] Several large randomized trials have been performed with diverse results[120-123] and meta-analyses have not been able to find any long-term improvement in incidence of BPD.[124,125] There has been an indication that late use of iNO to prevent BPD could be effective, but the effect size is likely to be small.[125] For the time being iNO is not recommended for routine use.[124,125]

Vitamin A

Vitamin A is necessary for lung growth and the integrity of epithelial cells of the respiratory tract. At birth Vitamin A levels are low in infants born preterm, which has been associated with an increased risk of BPD.[126] A Cochrane review by Darlow *et al* showed an improved survival without BPD in neonates with a birth weight < 1000 g with vitamin A supplement. The results were only marginal.[127] In a recent meta-analysis by Araki *et al*, a reduction of BPD was found in extremely low birthweight infants treated with vitamin A.[128] A drawback of vitamin A supplementation is that the most efficient way of administration is through intramuscular injection, which is painful for the infants. There are ongoing studies to evaluate efficacy on oral vitamin A.[129]

2.4.2.9 *Neonatal infections*

Infants born preterm are more sensitive to infections as their immune defence is not fully developed at birth. They have a thin, immature skin barrier, and they experience many invasive procedures. Chorioamnionitis increases the risk of early-onset sepsis. This leads to an inflammatory cascade. Late-onset sepsis also triggers a pro-inflammatory and pro-fibrotic response.[108, 111] Presence of microbes in lung fluid samples has been associated with the development of BPD.[130] Many studies have confirmed the association between coagulase-negative Staphylococcus, gram-negative bacteria, *ureaplasma spp* and BPD implying that the inflammation caused by bacteria is affecting lung development.[51,131,132]

2.4.3 Factors during childhood and adolescence

2.4.3.1 *Asthma*

Asthma is a common chronic inflammatory disorder of the airway that affects individuals of all ages. It usually starts in childhood and may persist into adulthood, but can develop at

any age. Asthma is characterized by airway obstruction and bronchial hyper-responsiveness. For more details see further in chapter 2.5.1. It is well known that asthma is not a single disease, but consists of different phenotypes with large variation in disease course and outcome.[133-138] In children born preterm with and without BPD, respiratory symptoms are often diagnosed as asthma though in many cases non-atopic. In early childhood these symptoms lead to an increased hospitalization rate and increase the use of inhaled corticosteroids and β 2 -agonists.[139, 140] Long-term follow-up of infants born <26 weeks gestation identified that 25% had an asthma diagnosis at 11 years of age while 56 % had evidence of abnormal spirometry.[141] Besides, the risk of asthma diagnosis was increased in children with BPD in infancy.[142] One hypothesis is that a family history of asthma increases the risk for development of BPD in preterm subjects and even predicts a route towards the more severe forms of BPD.[143,144]

2.4.3.2 *Physical activity*

Children born preterm may be more likely to have low physical activity if they have impaired lung function and decreased exercise capacity. Low levels of physical activity in children and adolescents have been associated with future health risks, including obesity, metabolic and cardiovascular disease.[145] There are few studies objectively measuring physical activity in children and adolescents born preterm but self-reported physical activity levels, especially those with BPD in infancy, have been reported to be lower than for term born children.[146] The Epicure study found no differences in physical activity when comparing children born preterm to term born controls using accelerometers.[147] As different methods have been used in the studies, comparison of results is difficult. In a meta-analysis on exercise capacity Edwards *et al* showed only a slight decrease in VO_{2max} comparing preterm born children with and without BPD to term born controls.[148] The long term importance of this modest reduction in work capacity remains to be further studied.

2.4.3.3 *Infections*

Several studies have shown that children who have lower respiratory tract illnesses in early life are at increased risk for subsequent chronic respiratory symptoms and decreased lung function which often persist into adult life.[149,150] In children born preterm Montgomery *et al* reported an association of asthma diagnosis at age five and respiratory infections during their first year in life.[142] Sigurs *et al* showed that a severe RSV infection before one year of age increases the risk of allergic asthma persisting into early adulthood.[151,152]

2.4.3.4 *Smoking and environmental factors*

Second hand smoking (SHS) has been associated with increased respiratory symptoms and reduced lung function in children. It is however difficult to separate the effects of pre- and postnatal exposure to tobacco smoke as many mothers smoking during pregnancy continue to smoke after giving birth.[153] Infants are at a greater risk of adverse effects of SHS since they have a high respiratory rate and immature lungs.[154] An increased risk of hospitalization due to respiratory disease in low birth weight children exposed to SHS but not exposed *in utero* has been demonstrated by Chen *et al*.[155] Kalliola *et al* showed increased FeNO levels and an increased airway resistance in preschool-aged children with multiple-trigger wheeze and exposed to SHS.[156] In a study of older school children SHS was associated with reduced lung function and higher frequency of self-reported respiratory symptoms and respiratory infections. Lung function measured by dynamic spirometry decreased with increasing numbers of smokers at home and higher ozone levels.[157] Exposure to air-pollution during infancy has been identified as a risk factor of lower lung function in childhood and adolescence.[158] The using of bio-mass fuel for cooking in the households is associated with more symptoms of severe asthma and lower lung function in children.[159,160]

2.5 CHRONIC AIRWAY OBSTRUCTION IN ADULTHOOD

2.5.1 Asthma

Asthma is defined by the Global Initiative for Asthma (GINA) as follows: "Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation".[161]

2.5.1.1 *Diagnosis*

The asthma diagnosis is established based on a history of respiratory symptoms and confirmed variable expiratory airflow limitation. The airflow limitation is often reversible, either spontaneously or with treatment. Different methods to measure lung function and airflow limitation can be used to confirm asthma diagnosis.[161] The recurrent episodes with symptoms and airflow limitation are often triggered by a variety of stimuli such as exercise, allergens or irritant exposure, change of weather, or respiratory infections. Symptoms may include wheeze, shortness of breath, chest tightness and cough, and fluctuate over time and in severity. More symptoms at night time and in the mornings are common. Children may express different symptoms compared to adults. In asthma, there should be a confirmed

airflow limitation including variability, assessed by clinical examination or preferable, measured by one or more lung function tests.[161,162]

2.5.1.2 *Pathophysiology and immunology*

The asthmatic airway inflammation includes numerous cell types, such as; mast cells, eosinophils, T lymphocytes, macrophages, neutrophils and epithelial cells.
The infiltration of inflammatory cells leads to bronchial hyper-responsiveness, and in the case of chronic inflammation, airway remodelling.[163] Recent molecular and genetic studies have identified clinical and inflammatory phenotypes that associate with specific biomarkers.[164] In allergic asthma the importance of the Th2 cells is characterized by the contribution of the Th2 cytokines IL-4, IL-5 and IL-13 that to leads to eosinophilic inflammation and IgE production. Innate lymphoid cell type 2 may also contribute to the production of IL-5 and IL-13.[165-167] Airway wall remodelling and repair are also important processes that could be driven by Th2 cytokines as well as by growth factors and cytokines derived from epithelial cells and macrophages.[168] Several patients have non-eosinophilic asthma, sometimes associated with neutrophilic inflammation. The neutrophilic inflammation is believed to be driven by a pathway where Th1 and Th17 cells play an important role.[169] The pathogenic mechanisms of neutrophil inflammation include an influx of neutrophils; IL-2, IL-8, IL-6, and IL-13 with release of interferon-γ, TNF-α, and several potent pro-inflammatory enzymes, such as myeloperoxidase, that cause tissue damage.[168,170] A "pauci-immune" phenotype has also been described, in which neither eosinophils nor neutrophils are seen in airway secretions.[171]

2.5.2 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is defined by the Global Initiative for chronic Obstructive Lung disease (GOLD) as follows:

"Chronic Obstructive Pulmonary Disease (COPD) is a common preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposures to noxious particles or gases."[172]

An underlying chronic inflammation; that causes structural changes, destruction of the lung parenchyma and narrowing of the small airways, is believed to drive the disease progress.[172] Tobacco smoking is the main risk factor for COPD but other environmental factors such as biomass fuel, air pollution, and occupational exposures may contribute.[173-176] Furthermore, host factors predispose individuals to develop COPD. These include

genetic alterations, e.g. alpha-1 antitrypsin deficiency[177]or polymorphisms in disease-susceptibility loci[178], infections, abnormal lung development [179-181], and events in early life such as preterm birth.[152,182-184] Dyspnoea and cough, sometimes with sputum production, are the most common symptoms of COPD. Periods with worsened symptoms are called exacerbations. Many patients suffer from comorbidities, in particular cardiovascular diseases, which increases the morbidity and mortality.[185]

2.5.2.1 *Diagnosis*

The diagnosis of COPD is based on the presence of symptoms and lung function measurements where a post-bronchodilator spirometry ratio of forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) below 0.7 is required for diagnosis.[172] The use of the ratio FEV_1/FVC may overestimate the prevalence of COPD in elderly subjects and underestimate the prevalence in younger subjects.[186] The use of lower limit of normal (LLN) could be helpful as a complement setting the diagnosis. The LLN is defined as the lower 5th percentile in a representative healthy population[186-188] COPD is further divided into four severity stages based on spirometry values for FEV_1 (Table 2).

Table 2. COPD severity degrees based on spirometry values according to GOLD[172]

Basal criterion for all stages: FEV_1/FVC < 0.7

Stage	Severity	FEV_1% of predicted
I	Mild	≥80
II	Moderate	50 - 79
III	Severe	30 - 49
IV	Very severe	<30

FEV_1 and FVC are based on post bronchodilator values. *Abbreviations*: FEV_1= forced expiratory flow volume in 1 second, FVC = forced vital capacity.

2.5.2.2 *Pathophysiology*

In chronic bronchitis an altered structure and function of the central airways is seen where the inflammation induces excessive mucus production which results in chronic cough. The mucus production is due to an increased number of goblet cells and enlarged submucosal glands caused by irritants.[189] Chronic bronchitis is a clinical diagnosis defined by cough

with mucus production during more than three months a year, for two consecutive years. Not all patients with COPD suffer from chronic bronchitis which also is a symptom occurring in individuals without COPD such as "healthy" smokers.[190] In bronchiolitis obstruction of the small airways develops. It is caused by an inflammation with repair and remodelling, fibrosis, thickening of the bronchial muscle layer and a production of exudates. Bronchiolitis results in reduction of FEV_1 and FEV_1/FVC ratio in patients with COPD. Obstruction of the smaller airways results in hyperinflation.[191,192] The altered structure of the lung parenchyma with destruction of alveolar walls leads to enlarged alveoli, emphysema. Emphysema results in reduced gas exchange and air trapping. The loss of lung elastic recoil due to emphysema causes impaired exhalation and can lead to carbon dioxide retention.[193] Pulmonary hypertension in COPD is due to effects on the pulmonary vasculature. Inflammatory cells contribute to the alterations of pulmonary vessels as well as hypoxia. However, pulmonary hypertension can be seen in early stages of COPD and is then more likely to be driven by inflammation rather than hypoxia.[194]

2.5.2.3 *Inflammation in COPD*

In COPD, a variety of exposures lead to airway inflammation where different cells and pathways are involved. The clinical course of COPD is partly a result of the interaction between the cells of the innate immune cells; such as macrophages, neutrophils and NK cells, and the adaptive immune system including $CD8^+$ T cells, $CD4^+$ T cells, and B cells.[18,195] Recruitment of lymphocytes is believed to play a central role in the pathogenesis of COPD. In patients with COPD elevated numbers of $CD8^+$ T cells both in central and peripheral airways and in the lung parenchyma have been shown.[196,197] Together with NK cells and NKT-like cells they damage the lung tissue through cytolytic activity.[198,199] $CD4^+$ T cells are more common in the severe stages of disease. They act by recruiting and activating other immune cells and thereby maintaining the inflammatory process.[18] Systematic inflammation in COPD, characterised with elevated levels of circulating leucocytes c-reactive protein (CRP), fibrinogen, TNFα and other biomarkers, have been associated with muscle weakness, cardiovascular disease, cancer and osteoporosis.[200-202]

2.5.3 COPD in never-smokers

At least 20 % of patients with COPD are never-smokers, but less is known about this group.[203,204] Since daily smoking is decreasing in the western world, COPD in never-smokers is likely to become a larger proportion of COPD in the future. It is therefore an urgent need to identify groups among never-smokers that have higher risk of COPD in order to target prevention.[205,206] Early life events and environmental exposures have been

suggested as possible causes of COPD in never-smokers, such as preterm birth, birth weight[207,208], growth pattern, maternal obesity, allergen exposure, respiratory infections, and genetic predisposition.[182,184] The mechanisms behind development of COPD due to such exposures *in utero*, in the neonatal period, and during childhood are still to be further investigated. Epigenetic studies (i.e. analyses of DNA modifications such as methylation that do not alter the sequence) are of high interest to explain how genes and early exposure might interact in the development of COPD in adulthood.[209] Other factors through life that has been identified as risk for later COPD are adult asthma, air pollution exposure, biomass smoke[176], occupational exposure[175], diet, recurrent respiratory infections, and tuberculosis. As many of these factors vary in the world there is diversity across nations in the prevalence and background of non-smoking related COPD. [152,182,210] In the multicentre international Burden of Obstructive Lung Disease (BOLD) study Lamprecht *et al* showed that never-smokers comprise a substantial proportion of individuals with COPD. Increasing age, female sex, a prior diagnosis of asthma and lower education levels are associated with an increased risk.[211]

3 AIMS

The overall aim in this thesis was:

- To study clinical, functional and mechanistic aspects of pulmonary outcomes in adolescents and young adults born preterm with and without BPD.

The specific objectives were:

- To evaluate the influence of BPD severity on lung function in a cohort of adolescents born preterm, and to evaluate development of lung function from 7 to 14 years of age using longitudinal spirometry and impulse oscillometry (IOS) data (Study I).
- To evaluate lung function and describe clinical characteristics at adult age in individuals born preterm with and without a prior diagnosis of BPD and compare with asthmatics and healthy controls (Study II).
- To characterize the inflammatory response both systemically and in the lower airways in adults born preterm with and without a history of BPD, and to compare with asthmatics and healthy controls (Study III).
- To investigate immune cells from the large airways of adults born preterm with and without a prior diagnosis of BPD, compare with asthmatics and healthy controls, and evaluate potential sex differences (Study IV).

4 STUDY SUBJECTS AND METHODS

4.1 STUDY SUBJECTS

The study-subjects included in this thesis are participants in the two different cohorts PALM II and LUNAPRE. Some subjects belong to both cohorts (Figure 4).

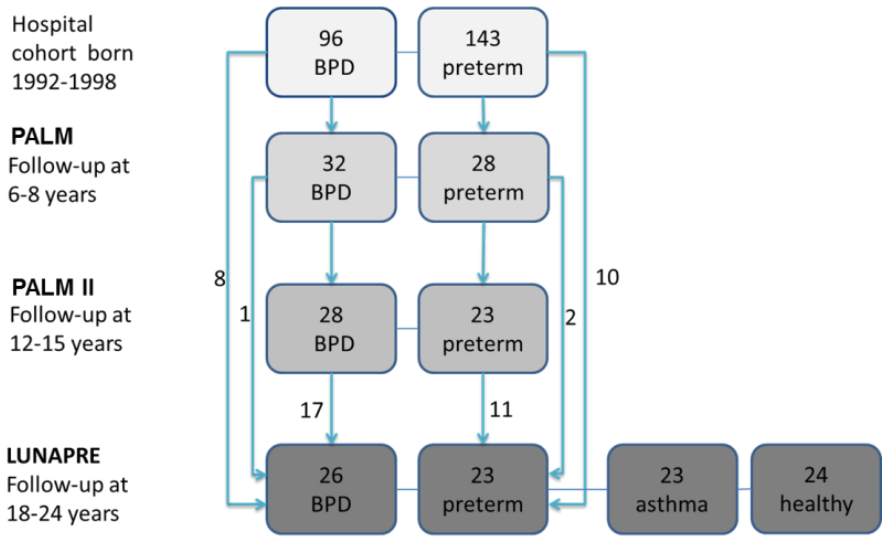

Figure 4. Schematic description of recruitment to the PALM-, PALM II-, and LUNAPRE-studies.

4.1.1 Study I

The PALM II study cohort consisted of 51 out of 60 individuals who participated in the study PALM (**P**sychology, **A**llergy, **L**ung function and **M**otor development) at six to eight years of age.[212] The study subjects were born before 32 weeks of GA and had been treated at the Neonatal Unit of Sachs' Children's Hospital, Stockholm, Sweden, between 1992 to1997. Twenty-three of the participants had been diagnosed with RDS but not BPD in the neonatal period, and 28 were graded as mild (n=17), moderate (n=7) or severe (n=4) BPD. They were examined in adolescence (at13-17 years of age) using spirometry, IOS, plethysmography, and ergospirometry. Comparison with lung function data from childhood (at 6-8 years of age) was also performed.[213]

4.1.2 Study II-IV

The majority of the data in this thesis was conducted within the LUNAPRE cohort (**LU**Ng obstruction in **A**dulthood of **PRE**maturely born; clinicaltrials.gov/ct2/show/ NCT02923648).[214] The LUNAPRE cohort consists of 96 young adults, 18-23 years of age, born between 1989 -1998 in Stockholm County. They were included in the study between

2013 and 2017. There were four study groups; healthy controls, subjects with mild allergic asthma, and subjects born preterm ≤ GA 32 weeks with and without BPD. The healthy controls and the subjects with mild allergic asthma were mainly recruited from the student website “Studentkaninen" (http://www.studentkaninen.se/) and through open study advertisements.

The LUNAPRE subjects born preterm were recruited from the PALM and PALM II cohorts, and from the original hospital cohort. With the exception of one person, all the participants in this group had been treated at the Neonatal Unit of Sachs’ Children and Youth Hospital, Stockholm, Sweden, between 1992 and 1998. In total, 53 individuals born preterm accepted participation, but four were excluded due to active smoking. All participants were non-smokers and without treatment with anti-inflammatory drugs (inhaled corticosteroids, leukotriene receptor antagonists, antihistamine) for a period of three months prior to inclusion. Information on lifestyle factors, environmental exposures, symptoms and health outcomes were obtained by questionnaires. Details on perinatal and neonatal history were collected from the Swedish Medical Birth Registry and medical charts. This included data on maternal smoking during pregnancy, multiple birth, caesarean section, treatment with prenatal steroids, Apgar score, GA at birth, BW, instillation of surfactant, number of days on a ventilator, CPAP and supplemental oxygen, retinopathy of prematurity (ROP) and patent ductus arteriosus (PDA). Small for gestational age was defined according to Marŝàl as -2SD.[215]

The participants were invited to four visits for the clinical examinations including: lung function measurements; dynamic spirometry, fractional exhaled NO (FeNO), IOS, multiple breath washout (MBW), static lung volumes, diffusing capacity for carbon monoxide (DL_{CO}) and methacholine challenge test; blood sampling, urinary sampling, high resolution computed tomography (HRCT), echocardiography; bronchoscopy with biopsies, bronchial brushings, bronchial wash, and collection of BAL fluids (Figure 5).

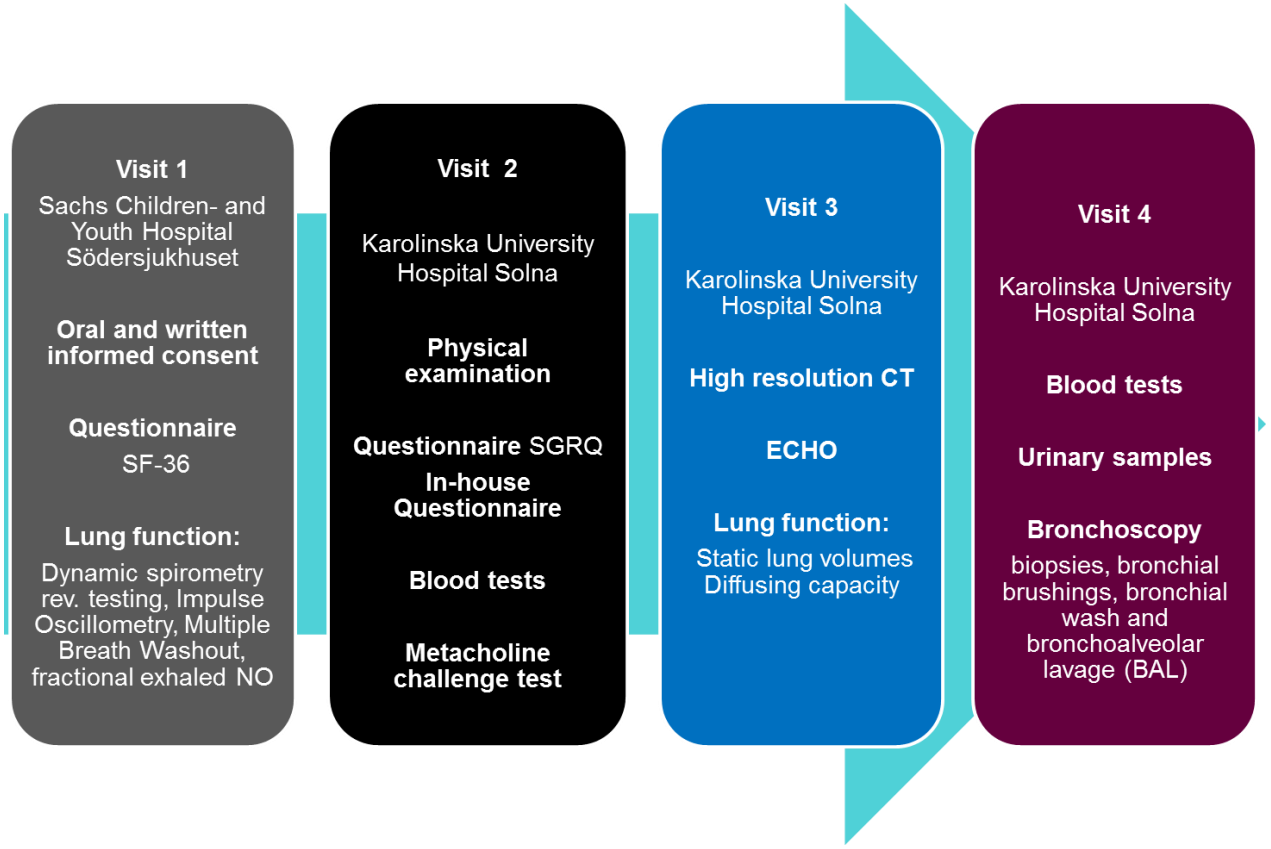

Figure 5. Flow chart of the participants' way though the LUNAPRE study protocol (study II-IV).

4.2 METHODS

4.2.1 Questionnaires

Three different questionnaires were used in the LUNAPRE study.

a) SF36

Health-related quality of life (HRQoL) was assessed using the Swedish version of the short form health survey (SF)-36 (first version), a self-administered 36-item questionnaire covering eight different domains: physical functioning, physical role functioning, bodily pain, general health, vitality, social functioning, emotional role functioning, and mental health. Raw points are transformed into a score from 0 to 100 for each dimension, with 100 reflecting the best possible HRQoL. The scores are summarized into two aggregated measures: the physical (PCS) and mental (MCS) component summary scores.[216]

b) St George's Respiratory Questionnaire (SGRQ)

SGRQ is a disease specific 50-item questionnaire designed to measure impact on overall health, daily life, and perceived well-being in patients with obstructive airways disease.

Scores are calculated for three domains: symptoms, activity and impacts (psycho-social) as well as a total score. A higher score means a poorer HRQoL. A minimum change in score of four units was established as clinically relevant after patient and clinician testing. The SGRQ has been used in a range of settings such as randomized controlled therapy trials and population surveys.[217-219]

c) An "in-house" questionnaire designed for the LUNAPRE study with questions on environmental exposures, education, lifestyle, tobacco use, former and present health conditions, symptoms, and pharmacological treatment.

4.2.2 Lung function assessment

4.2.2.1 *Dynamic Spirometry*

Dynamic spirometry is the most used and established lung function test in both clinical settings and research. Forced vital capacity (FVC) is defined as the maximal volume of air exhaled with maximally forced effort from a full inspiration. Forced expiratory volume in one second (FEV_1) is the maximal volume of air exhaled during the first second. FVC reflects the size of the lung and FEV_1 the flow dimensions (Figure 6). The ratio FEV_1/FVC gives an indication of the relative size of the airways compared with the lung volumes. A low ratio indicates airway obstruction while a high ratio implicates a restrictive pattern in presence of a low FVC.

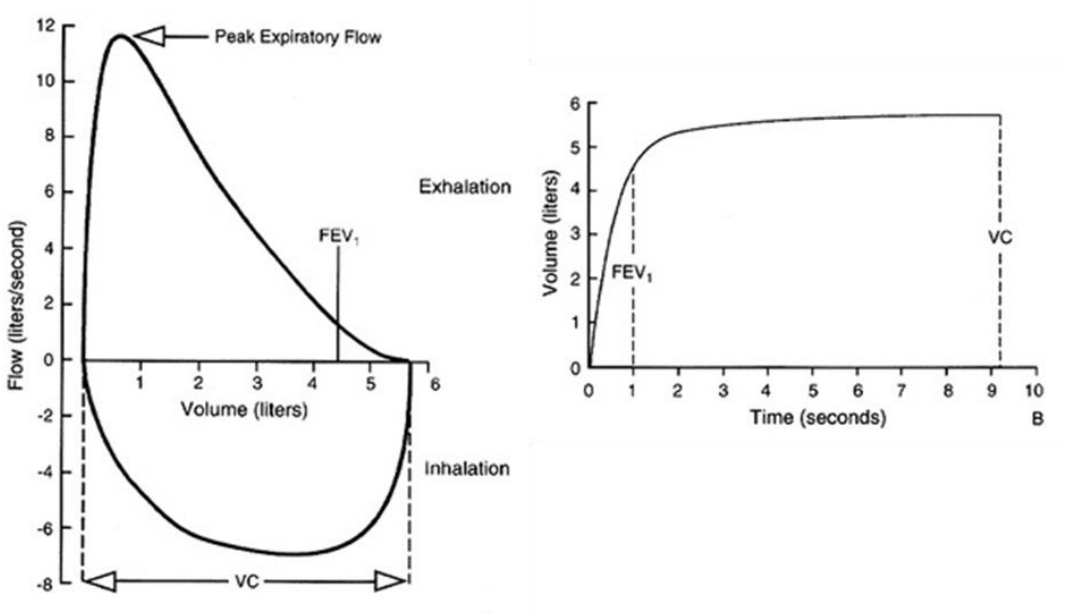

Figure 6. Dynamic spirometry volume flow loop. FEV_1: Forced expiratory volume in one second. Reproduced from Crapo RO[220]. With permission from The New England Journal of Medicine.

A bronchodilator responsiveness test is used to evaluate the potential reversibility of an airway obstruction. Dynamic spirometry before and after inhalation of a β-2-agonist is compared, an increase of at least 12% and 200 mL in FEV_1 is considered a significant improvement. In some cases, an improvement in FVC can also be seen as an effect of decreased residual volume when small airways open up.

Dynamic spirometry in PALM II and LUNAPRE was measured according to American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines[221], using the Sensormedics 6200 body plethysmograph (SensorMedics; Yorba Linda, CA, USA). Examination was performed with the subject in sitting position and wearing a nose clip. Each subject performed at least three acceptable forced vital capacity expirations. The highest values of FVC and FEV_1 were registered.

4.2.2.2 *Lung volumes and diffusion capacity for carbon monoxide*

Static lung volumes are measured using methods in which airflow velocity is unimportant. Tidal volume (V_T) is the volume of air that is inhaled or exhaled with each breath. Inspiratory reserve volume (IRV) is the maximal volume of air that can be inhaled from the V_T end-inspiratory level. Expiratory reserve volume (ERV) is the maximal volume of air that can be exhaled after a normal tidal exhalation. Residual volume (RV) is the volume of gas remaining in the lung at the end of a maximal expiration. Inspiratory capacity (IC) is the maximal volume of air that can be inhaled from the V_T end-expiratory level. Vital capacity (VC) is the volume change between maximal inspiration and maximal expiration. The functional residual capacity (FRC) is the volume of air present in the lung at the end expiration during normal breathing. FRC can be measured by body plethysmography, gas washout or gas dilution tests, and by radiograph. Total lung capacity (TLC) is the volume of air in the lung at the end of a maximal inspiration. It is usually calculated in one of two ways: (1) TLC = RV + VC or (2) TLC = FRC + IC. The method of measurement (i.e., gas dilution, body plethysmography) should be specified (Figure 7).[222]

The diffusing capacity test aims to determine the diffusion of gas between the alveoli and the lung capillary. The most common way to measure the diffusing capacity in the lungs (DL) is by using carbon monoxide (CO). The DL can be impaired due to small diffusion area, large diffusion way or low uptake to the blood of CO; e.g. due to low haemoglobin concentration. Impaired DL_{CO} is common in patients with COPD but normal or increased in patients with asthma.[223]

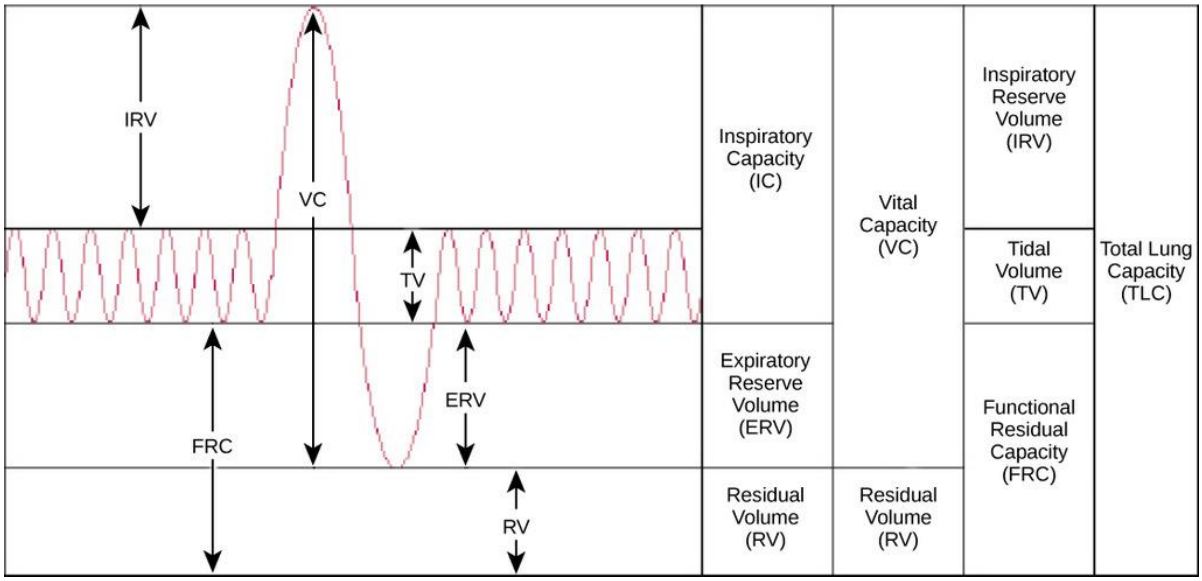

Figure 7. Static lung volumes and capacities based on a volume-time spirogram of vital capacity (VC). TLC: total lung capacity, IRV: inspiratory reserve volume, TV: tidal volume, FRC: functional residual capacity, RV: residual volume, ERV: expiratory reserve volume, IC: inspiratory capacity

In the studies included in this thesis, static lung volumes were measured by whole body plethysmography according to ATS/ERS guideines.[221,224] In PALM II, testing took place at the Department of Clinical physiology, Södersjukhuset, using the Sensormedics 6200 body plethysmograph (SensorMedics; Yorba Linda, CA, USA). Mean values of TLC, FRC and RV were registered. In LUNAPRE, measurements were performed at the Department of Clinical physiology, Karolinska University Hospital, using the Vmax62 J CareFusion (SensorMedics, Yorba Linda, CA, USA). Measures included VC, FRC, TLC and RV, as well as DL_{CO}. The diffusing capacity was corrected for haemoglobin levels.

4.2.2.3 *Multiple breath washout*

Inert gas washout provides information of ventilation inhomogeneity and gives information about small airway function. It can be performed as single breath or multiple breath washout (MBW). Single breath washout requires a full vital capacity breath while MBW is measured during tidal breathing and therefore more feasible as it needs less cooperation and coordination. One common and robust parameters of ventilation inhomogeneity is the lung clearance index (LCI) which is the number of the lung volume turnovers to clear out the inert gas measured to $1/40^{th}$ of the starting concentration. As LCI is independent of age, weight and height it is ideal for longitudinal monitoring both in clinic and research.[225,226]

In LUNAPRE nitrogen (N_2) MBW was performed using the Exhalyzer®D N_2 MBWdevice (Eco Medics AG, Duernten, Switzerland). Two technically acceptable N_2 MBW tests were

performed in accordance with recently published studies.[225,227] Mean values for LCI were extracted.

4.2.2.4 *Impulse Oscillometry*

Involvement of the small airways may be present in lung diseases such as BPD, COPD, and asthma. This part of the airways is difficult to measure and is sometimes referred to as "the quiet zone" of the lung.[228] As impulse oscillometry (IOS) only requires tidal breathing and no forced manoeuvers it is suitable for patients with difficulties to produce enough force needed for dynamic spirometry. The data obtained from IOS is thought to represent compound functions of the lung such as small airway obstruction and airway mechanics, which includes the elastic properties of the lung.[229,230]

In both PALM II and LUNAPRE, assessment was accomplished using the Jaeger MasterScreen-IOS system (CareFusion Technologies, San Diego, CA, USA). In summary, pressure impulses were sent from a loudspeaker through the respiratory system. The study subjects were requested to breathe tidal breathing with the lips tightly sealed around the mouthpiece and supporting cheeks with their hands to avoid impulse pressure loss due to upper airway shunt. After quality inspection, the mean value of resistance at 5 and 20 hertz (R_5, R_{20}), frequency dependence of resistance (R_{5-20}) and the area of reactance (AX) were used for analysis.[231-234]

4.2.2.5 *Methacholine challenge test*

The methacholine challenge testing is one method of assessing airway responsiveness. Bronchial challenge with direct stimuli, such as methacholine, is very sensitive for diagnosing asthma patients. Direct stimuli cause bronchoconstriction acting on the effector cells, such as airway smooth muscle cells, bronchial vascular endothelial cells and mucus-producing cells. However, these stimuli lack specificity, both in differentiating asthma from normal and asthma from bronchial hyper-responsiveness seen in other diseases, such as COPD, congestive heart failure, cystic fibrosis, bronchitis, and allergic rhinitis.[235] However, a negative test has a high negative predictive value and is likely to rule out asthma. The challenge is performed by repeated inhalations of methacholine followed by lung function testing. When a 20 % fall in FEV_1 is achieved, the test is stopped and a concentration or dose is calculated and named provocative concentration (PC) or provocative dose (PD). The results are reported as a percent decrease in FEV_1 from baseline (or post diluent if a diluent step is used). A single number, PC_{20} or PD_{20}, may be used to summarize the results for clinical purposes.[236-238]

In LUNAPRE bronchial hyper-responsiveness to methacholine was assessed utilizing a Spira nebuliser (Spira Elektro 2, Respiratory Care Centre, Hämeenlinna, Finland) according to modified protocol[239] at the inclusion and was the criterion used for diagnosis of asthma.[240]

4.2.2.6 *Fractional exhaled nitric oxide*

In respiratory epithelial cells, inflammatory cells and vascular endothelial cells nitric oxide is synthesized in the catalysis of nitric oxide synthase.[241] Fractional exhaled nitric oxide (FeNO) is a marker of eosinophilic airway inflammation and of the total number of airway inflammatory cells. Elevated level of FeNO has been documented in asthma and is used clinically to monitor airway inflammation in asthma, and to evaluate corticosteroid responsiveness.[242,243]

In LUNAPRE FeNO was measured with a chemiluminescence analyser (ECO MEDICS EXHALYZER® CLD 88sp with DENOX 88, ECO MEDICS, Dürnten, Switzerland) The procedure was performed in accordance with published guidelines.[244] Mean exhalation flow rate was 50 mL/s ± 10% during the NO plateau. The manoeuvre was repeated until two exhalations agreed to within 5% coefficient of variation, or three exhalations agreed to within 10% coefficient of variation. The NO concentration, FeNO, was defined as the mean of these values expressed in parts per billion (ppb). The analyser was calibrated using a standard NO calibration gas (Air Liquide Deutschland GmbH, Krefeld, Germany). In a few study subjects FeNO was measured using a NIOX device (Aerocrine AB, Solna, Sweden).[245]

4.2.2.7 *Ergospirometry*

Ergospirometry is a diagnostic procedure to continuously measure respiration and gas metabolism during ergometer exercise. It measures the maximal oxygen consumption (VO_2max), the amount of oxygen that is consumed for energy at maximum effort. VO_2max is an indicator of the functional capabilities of the systems involved in the transfer of oxygen from the air, further from the lungs into the blood, transfer of blood to the muscles, and finally the use of oxygen in the muscles. It enables evaluation of function and exercise capacity of the cardiopulmonary system and metabolism. Ergospirometry is useful to help evaluate breathlessness, but also in the diagnostic process of myocardial ischemia, COPD, pulmonary embolism, exercise-induced asthma, unfitness, hyperventilation syndromes and many more.[246,247]

In PALM II, ergospirometry was performed using an incremental Monark cycle ergometer (Electronic Ergomedic 839E, Monark Exercise AB, Vansbro, Sweden). The study subjects used a mouthpiece and wore a nose clip. Heart rate was monitored continuously. Minute ventilation, oxygen output (VO_2) and carbon dioxide output were measured and calculated from a mixing chamber every 30 seconds using a SensorMedics Vmax Encore (SensorMedics, Yorba Linda, CA, USA). The study subjects were advised to start pedalling at 60-70 revolutions/min. After three minutes of unloading cycling, load was increased every minute by 15W. The study subjects were supported to cycle as long as possible. Peak values for all variables were gathered by averaging data over the last 20 seconds of maximum completed work. Peak VO_2 in ml/kg/min was predicted using formulae for healthy subjects.[148,248,249]

4.2.3 Bronchoscopy and analysis of inflammatory cells

4.2.3.1 *Bronchoscopy*

Bronchoscopy is a diagnostic procedure for routinely clinical use in several lung diseases. This procedure enables direct inspection and sampling of the airways. A small camera on the tip of the bronchoscope is used for viewing the airways, photos can be taken and filming can be done. Samples from the lower respiratory tract can be obtained by bronchoalveolar lavage (BAL). From the central airways, bronchial brushings, mucosal biopsies, and bronchial wash can be carried out (Figure 8).

Bronchoscopy was performed in the study subjects in LUNAPRE according to a standard protocol.[250] The study subjects received pre-medication to be relaxed during the procedure. A flexible bronchoscope was inserted into the airways through the nose. It was wedged into a middle lobe bronchus where five aliquots of 50 mL each of sterile phosphate-buffered saline solution were instilled and recollected as BAL fluid. Multiple mucosal biopsies were and bronchial brushings were performed from central parts of the airways. In addition, instillation of three portions of 10 mL phosphate-buffered saline each was performed for sampling of the large airways designated bronchial wash.

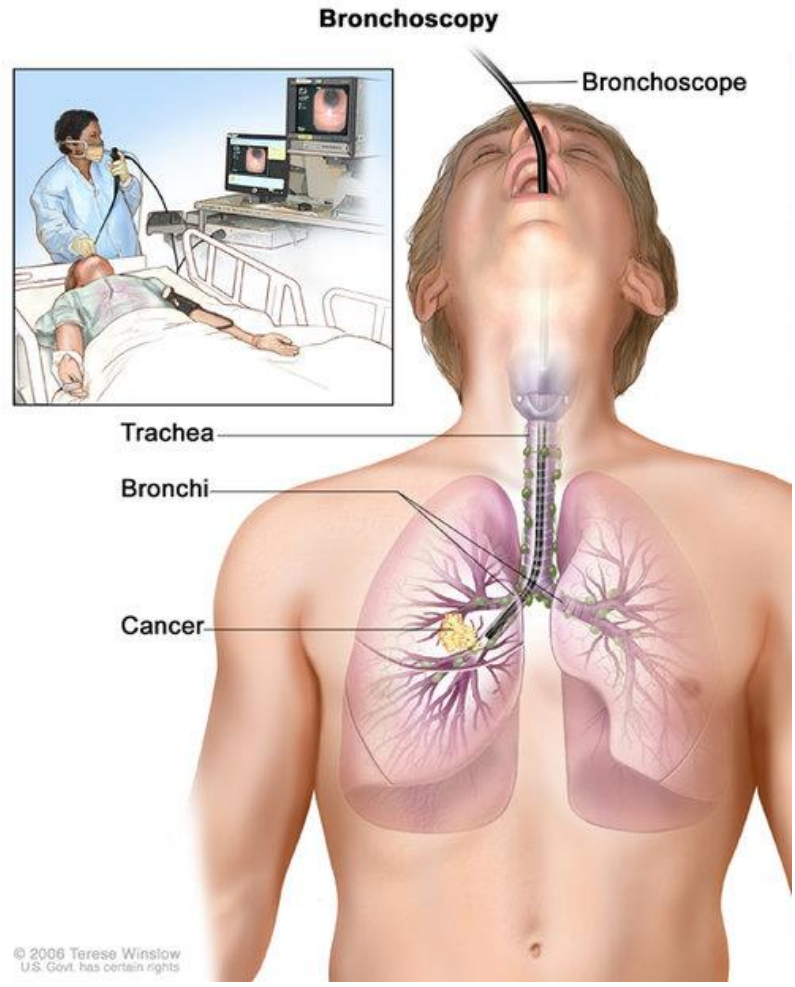

Figure 8. Bronchoscopy. Credit: The National Cancer Institute©2006 Terese Winslow, LLC, U.S. Govt. has certain rights

4.2.3.2 *Analysis of inflammatory cells from bronchoalveolar lavage*

The volume of BAL fluid was measured and debris and mucus were removed by filtration. The BAL cells were separated by centrifugation. Cell differential cell counts were determined on cytospin slides prepared with native pellet and stained with May-Grünwald Giemsa. In these cytospin slides, 500 cells were counted. Mast cells were stained with toluidine blue, and the number of cells within 10 visual fields (16 x magnifications) was scored and reported as absolute number of these cells. The cell-free BAL fluid was centrifuged to eliminate cell debris and the supernatant was stored at -80˚C until analysis.

4.2.3.3 *Multicolour flow cytometry*

Flow cytometry is a technique that is used for measuring many characteristics of individual cells in large amounts. The sample of cells in suspension passes through a laser beam under a laminal flow. The cells have often been labelled with fluorescent monoclonal antibody markers prior to infusion into the instrument. The size, the granularity and the fluorescent monoclonal antibody markers are absorbed by the laser beams and deflect different

wavelengths. A large number of cells can be measured rapidly and the data gives detailed information on the cell types. There are more than 350 different clusters of differentiation (CD) which is the international nomenclature to classify antibodies against epitopes on leukocytes in order to identify surface molecules.

In LUNAPRE three different panels of monoclonal antibodies were used to characterize the major lymphocyte subsets and the T-cell differentiation subsets in BAL (Table 3). Lymphocyte subsets in BAL were analysed using eight colour flow cytometry (FACSCanto II; BD Medical, Franklin Lakes, NJ, USA). Data were processed in FACSDiva 6.1.2 (BD Medical). Flow cytometric data were excluded from the data analysis if fewer than 50 events were detected in the final gate (Figure 9).

Table 3. Three panels of monoclonal antibodies were used to characterize lymphocytes in BAL.

Panel 1 T cells		Fluorochrome	Panel 2 NK cells & Tregs		Fluorochrome	Panel 3 B cells & neutro/eos		Fluorochrome
CD45	Leukocytes	APC-H7	CD45	Leukocytes	APC-H7	CD45	Leukocytes	APC-H7
CD3	Lymphocytes	FITC	CD3	Lymphocytes	FITC	CD19	B cells	Brilliant violet
CD8	Cytotoxic T cells	v450	CD8	Cytotoxic T cells	v450	CD23	Mature B cells	PerCP-Cy5.5
CD4	T helper cells	Brilliant violet 421	CD56	NK cells	PE	CD27	Resting memory B cells	PE-CF594
CD45RO	Memory/Naïve T cells	PE-CF594	CD16	NK cells, macros & neutros	APC	CD66b	Granulocytes	FITC
CD45RA	Active T cells	PE	CD4	T helper cells	Brilliant violet 421	CD16	NK cells, macro-phages & neutrophils	v450
CD27	Degree of activation	PerCP-Cy5.5	CD127	Memory/Naïve T cells	PerCP-Cy5.5	CD62L	L-selectin.	PE
CD69	1-2 hours. Early activation	APC	FoxP3	Transcription factor	PE-CF594	CD49d	eosinos	APC

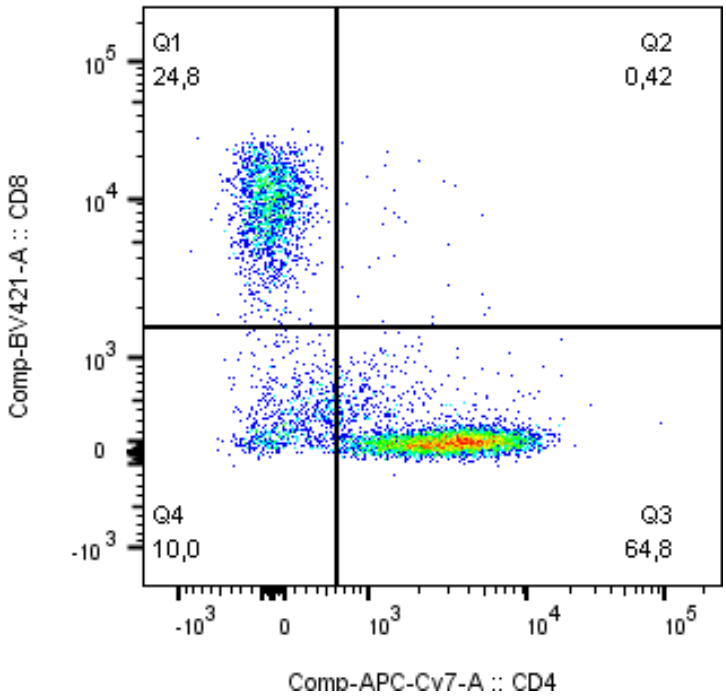

Figure 9. Representative flow cytometric dot plot of $CD8^+$ (y-axis) and $CD4^+$ (x-axis) among $CD3^+$ T cells from BAL.

4.2.3.4 *Analysis of inflammatory cells in bronchial wash*

In order to collect cells from the large airways 3 x 10 mL phosphate-buffered saline was instilled during bronchoscopy in three different segments in the right upper lobe which immediately was recovered by gentle suction. Cells in bronchial wash were scored on cytospin slides stained with May-Grünwald Giemsa.

4.2.4 Peripheral blood tests

In LUNAPRE peripheral blood was drawn for analysis in the routine laboratory of Karolinska University Hospital, Solna, Sweden for white blood cells with differential counting, platelets, red blood cells, haemoglobin, creatinine, systemic inflammatory markers (acute-phase proteins: CRP, alpha-1 antitrypsin, orosmucoid, albumin and haptoglobin) and Phadiatop® (Phadia /Thermo Fisher Scientific, Uppsala, Sweden). Phadiatop® screens for IgE antibodies to a mix of common aeroallergens: birch, timothy, olive tree and mugwort, cat, dog and horse dander, mold (*Cladosporium herbarum*), and house dust mite (*Dermatophagoides pteronyssinus*). Additional blood samples for research purposes were taken at the final visit prior to bronchoscopy.

4.2.5 Statistical analyses

Analyses were performed with the Stata 13.1 software package (StataCorp LP, College Station, TX, USA) or the SPSS 25 and Prisma 8. In all studies, a P-value <0.05 was considered to be statistically significant. Demographic data were presented as median and range for continuous variables, or numbers and percentages for categorical variables. In the case of non-normally distributed data, comparisons between groups were performed using the Wilcoxon rank-sum test for continuous variables. The Pearson's χ-squared test was used for categorical outcomes. FVC, FEV_1 and FEV_1/FVC were converted to z-scores using the Global Lung Initiative reference values (GLI).[251] The lower limit of normal was defined using z-scores below -1.64 (<5th percentile).

4.2.5.1 *Study I*

Cross-sectional and longitudinal comparisons between groups were made using the Wilcoxon rank-sum test. Trends across BPD severity groups were assessed using the nonparametric test developed by Cuzick for trend across ordered group.[252] Associations between other lung function outcomes and BPD severity groups were analysed using linear regression on the median, adjusting for sex, height and age when appropriate. The influence of treatment with prenatal and postnatal steroids, surfactant, ROP, PDA, necrotizing enterocolitis, septicaemia and maternal smoking on the relationship between BPD and lung function outcomes was evaluated with linear regression on the median in a univariate and stepwise manner. For longitudinal analysis of impulse oscillometry data, mixed models was used. Time-dependent covariates included in the model were height and age. BPD group and sex were time-invariant covariates. To assess potential variations of the effect of BPD on lung function over time, an interaction term between time and BPD group was included in the model.

4.2.5.2 *Study II-III*

Associations between BAL variables and phenotype (BPD-, preterm-, asthma- or healthy control) groups were analysed using linear regression on the median, adjusting for sex, height, and age when appropriate [253]. In study III, first the BPD group was compared with the preterm group, asthmatics and healthy controls separately. Secondly, the BPD- and preterm groups were combined and compared with healthy controls to evaluate overall associations with preterm birth regardless of BPD status. Correlations were assessed with Spearman's test. Correction for multiple testing was performed with false discovery rate by Benjamini-Hochberg. A FDR of < 0.05 was considered statistically significant.

4.2.5.3 *Study IV*

Comparisons between groups were evaluated by the Kruskal–Wallis test followed by the Mann–Whitney U test, when appropriate. Subgroup analyses for sex were performed. The suitable continuous variables were further analysed by the area under the curve (AUC) statistics for distinguishing the sub-groups. Spearman's rank correlation was used to evaluate the associations of lymphocyte and eosinophils proportion with other variables.

4.2.6 Ethical approvals

All studies were approved by the Regional Ethical Review Board in Stockholm:
Study I (PALM II): 2007/767-31/2.
Study II-IV (LUNAPRE): 2012/11872-31/4 with amendments: 2013/1416-32 and 2017/868-32, and by the Radiation Protection Committee Karolinska University Hospital, Solna K2641-2012.

5 RESULTS

5.1 STUDY I

Adolescents with a history of BPD had lower FEV_1 compared to those without BPD (-0.61 vs.-0.02 *z-scores*, *P* <0.05), with a trend for decreasing FEV_1 values with increasing BPD severity (*P* for trend 0.002). Seventy-five percent of the individuals with severe BPD had FEV_1 below the lower limit of normal (LLN; -1.64 z-scores) both in childhood and adolescence, and 43% of the individuals with moderate BPD (Table 4) in both time periods. Peripheral airway involvement in the severe BPD-group was indicated by higher frequency dependence of resistance, R_{5-20} (*P* <0.001 vs. non-BPD subjects). No differences between the groups were found for TLC or RV. There was a non significant tendency towards less working capacity with increasing BPD severity.

Table 4. Percentage of individuals with spirometry measures below the lower limit of normal (-1.64 z-scores), at childhood and adolescence (numbers and percentage).

	Childhood (7 years)				**Adolescence (14 years)**			
	No BPD	**Mild BPD**	**Moderate BPD**	**Severe BPD**	**No BPD**	**Mild BPD**	**Moderate BPD**	**Severe BPD**
FEV_1	0	12 (75 %)	6 (86 %)	4 (100 %)	0	3 (23 %)	2 (29 %)	4 (100 %)
FVC	0	3 (19 %)	0	3 (75 %)	0	2 (15 %)	0	0
FEV_1/FVC	4 (17 %)	3 (19 %)	3 (43 %)	3 (75 %)	2 (10 %)	2 (15 %)	3 (43 %)	3 (75 %)

Between childhood and adolescence, FEV_1/FVC *z-scores* decreased in all groups and particularly in the severe BPD group (from -1.68 *z-scores* at childhood to -2.74 *z-scores* at adolescence, *P* <0.05 compared to the non-BPD group (Figure10).

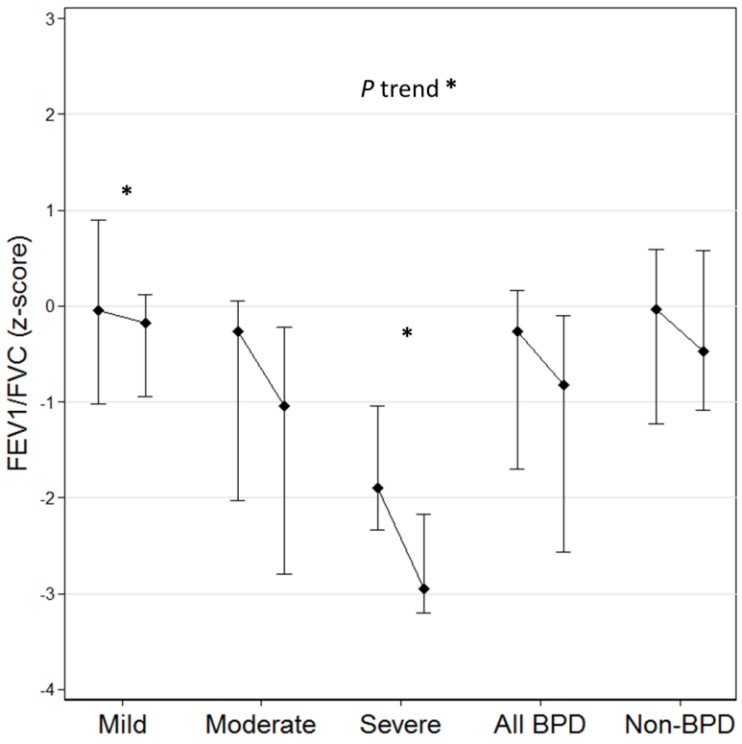

Figure 10. Z-scores presented as median (25th and 75th centiles) for FEV_1/FVC at childhood and adolescence in mild-, moderate-, severe-, all BPD-, and non-BPD groups. *P*-values for evaluation of the evolvement from childhood to adolescence in mild, moderate and severe BPD groups compared to the non-BPD group. Nonparametric test for trend across ordered groups comparing mild-, moderate- and severe BPD-groups to the non-BPD group. *: $P<0.05$

5.2 STUDY II

Dynamic spirometry showed a significant reduction in the FEV_1, FVC and FEV_1/FVC ratio in subjects born preterm with BPD compared to all other groups (Figure 11). Further, ventilation inhomogeneity was indicated by an increased LCI in the BPD-group. Both preterm groups showed decreased DL_{CO} compared to healthy controls and the asthma group. The only difference in lung function parameters between the asthmatic and healthy group was a higher mean FeNO in the former.

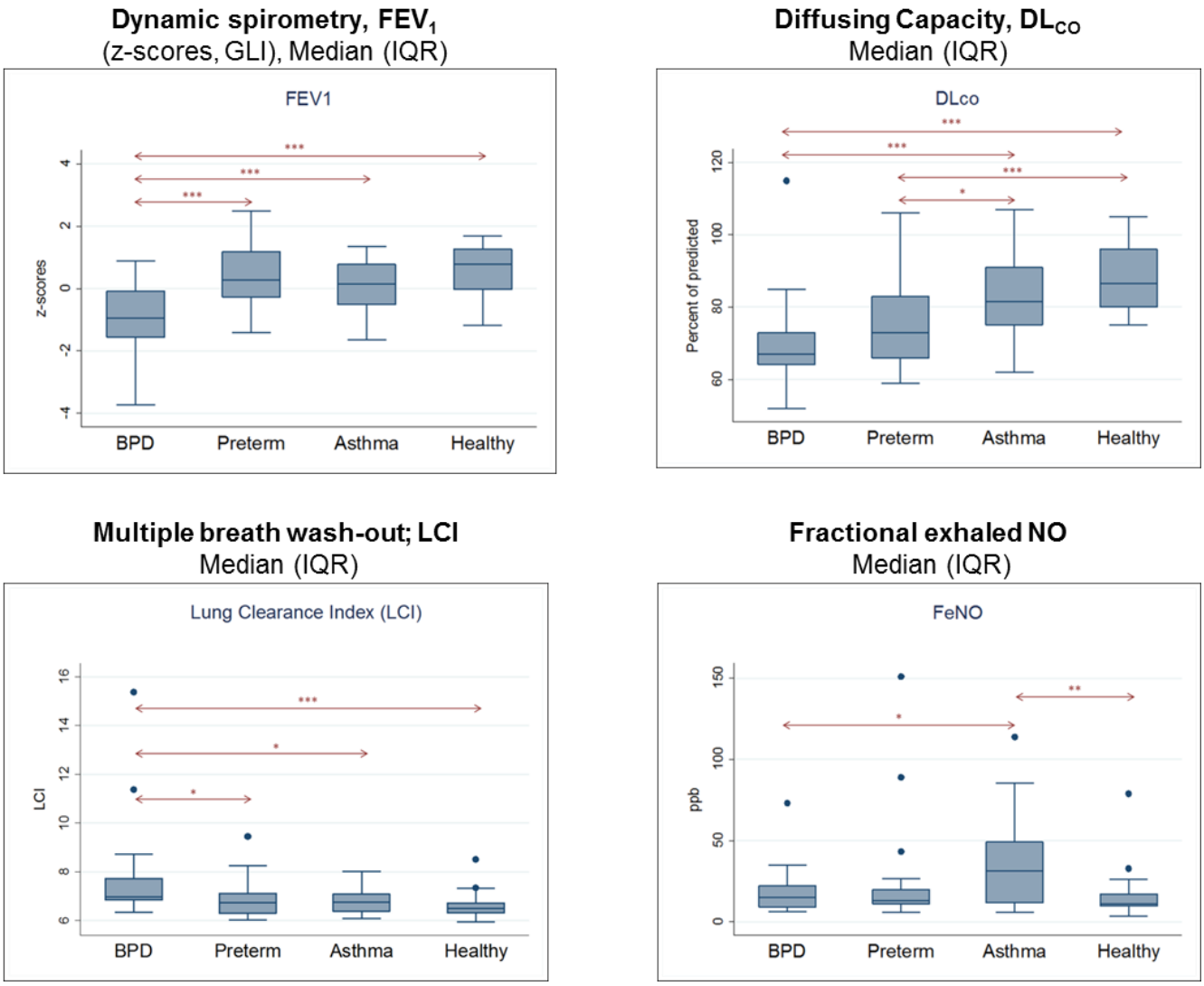

Figure 11. Graphical summery of airflow, gas diffusion, ventilation inhomogeneity and airway inflammation. The boxplots show the median values (IQR) for FEV1, DL_{CO}, LCI, and FeNO. *: p <0.05; **: p <0.01; ***: p <0.001

Table 5. Proportions of subjects in each group with spirometry measures below the lower limit of normal (-1.64 z-scores; numbers and percentage).

	Healthy n=24	Asthma n=23	Preterm n=23	BPD n=26
FEV_1 z-scores	0	0	0	5 (19) *
FVC z-scores	0	0	1 (4.3)	0
FEV_1/FVC z-scores	0	1 (4.3)	1 (4.3)	7 (27) **

BPD-, preterm- and asthma-groups were compared to healthy controls. Data are presented as numbers (%). FEV_1, FVC, and FEV1/FVC: post bronchodilator measures. *: p <0.05; **: p <0.01; ***: p <0.001. Abbreviations: BPD: bronchopulmonary dysplasia; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity

Further, in the BPD group 19 % had FEV_1, and 27% had FEV_1/FVC below LLN (Table 5).

The asthmatic group reported more physical symptoms than the healthy control group in both the SF-36 and SGRQ questionnaires. The preterm group reported more physical symptoms than healthy control group only in the SF-36. There was no difference between the BPD group and the healthy controls regarding physical symptoms measured by either questionnaire. Both preterm groups scored lower than healthy controls in the mental component of the SF-36 (Table 6).

Table 6. Overview of HRQoL questionnaires.

	Healthy	Asthma	Preterm	BPD
SGRQ Total score	3.8 (2.0; 6.0)	16.5*** (9.2; 24.4)	4.9 (3.3; 8.1)	6.3 (3.4; 11.6)
SF-36 PCS (physical component score	57.6 (55.7; 58.4)	53.5** (49.4; 55.1)	52.8* (49.4; 54.1)	56.3 (53,2; 57.6)
SF-36 MCS (mental component score)	51.7 (48.3; 53.9)	48.3 (40.6; 53.2)	44.2** (40.6; 52.5)	48.2* (40.4; 50.8)

BPD-, preterm-, asthma- groups were compared to healthy controls. Data are presented as median (IQR). *: p <0.05; **: p <0.01; ***: p <0.001

5.3 STUDY III

No statistical differences in BAL cell differential counts were observed between the four study groups. The majority of cells in BAL fluid were macrophages (median 90.8% [IQR 84.4 – 94.2]) and lymphocytes (8.0% [IQR 5.0 – 14.6]). The BPD group had a higher proportion of $CD8^+$ T cells and a lower proportion of $CD4^+$ T cells resulting in a lower CD4/CD8 ratio compared to healthy control group (Figure 12). The BPD group had increased proportion of $CD69^+CD8^+$ T cells, i.e. activated $CD8^+$ T cells when compared to the preterm group. Among the $CD4^+$ T cells in BAL, effector memory cells ($CD27^-$ $CD45RA^-$) were the dominant subset, followed by central memory cells ($CD27^+CD45RA^-$). The preterm group displayed lower percentages of central memory and naïve T cells ($CD27^+CD45RA^+$) compared to healthy controls and a higher percentage of effector T cells ($CD27^-CD45RA^+$) compared to the asthmatics. The BPD group had lower proportion of Treg ($CD4^+$ $FoxP3^+$) compared to the asthmatics. The proportion of NK cells ($CD3^-$ $CD16^+$ and/or $CD56^+$) was lower in BAL from the preterm group compared to the BPD group. There were no differences between the groups in the percentage of NKT-like cells ($CD3^+$ $CD16^+$ and/or $CD56^+$). All groups displayed low levels of B cells.

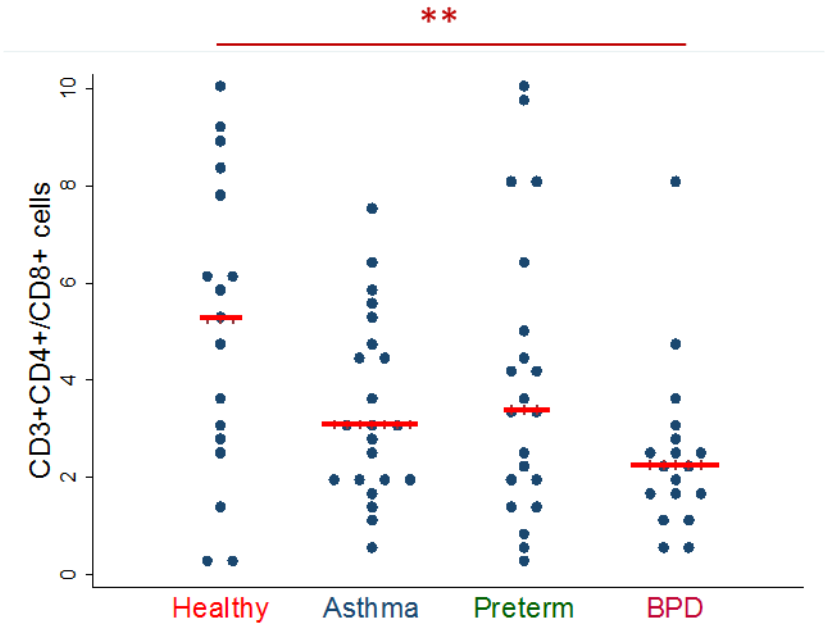

Figure 12. The dot plot show median values for CD4/CD8 ratio in BAL. **: p <0.01

Further, $CD4^+$ T cells correlated positively and $CD8^+$ T cells negatively with expiratory flow rates in the preterm born subjects (Figure 13).

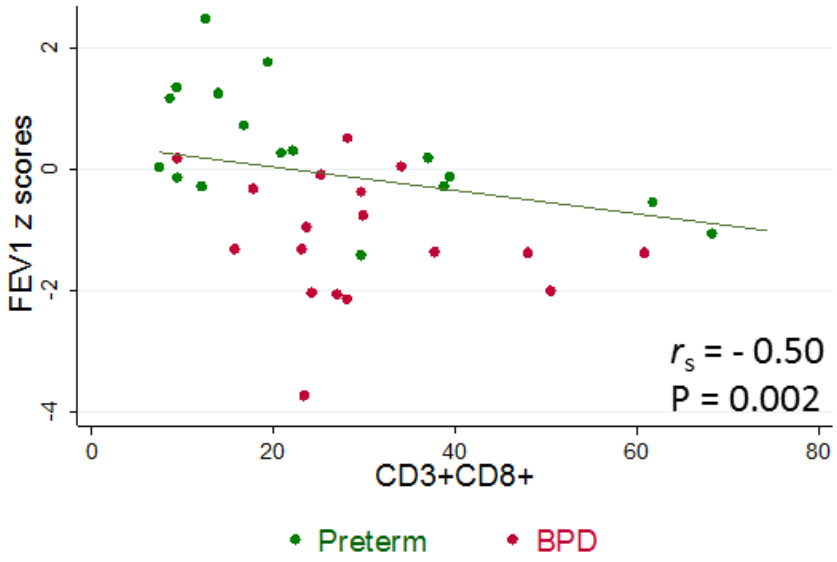

Figure 13. Correlation in both preterm groups (preterm + BPD) between $CD8^+$ T cells (%) and FEV_1 z scores. r_s: Spearman rank correlation coefficient

5.4 STUDY IV

The total number of cells in bronchial wash did not differ between the four groups. The most common cell type was epithelial cells (54.8.5% [IQR 49.2-65.6] in both preterm groups and 61.0% [IQR 48.5-74.4] in the asthma- and healthy groups). The proportion of eosinophils was higher in asthmatics compared with the other three groups. The lymphocyte proportion in bronchial wash was elevated in the BPD group (6.6% [IQR 5.0 - 8.0]) when compared to the asthma- and healthy groups (3.4% [IQR 3.2-5.4] and 3.8% [IQR 2.4-4.8], respectively), as seen in Figures 14 and 15.

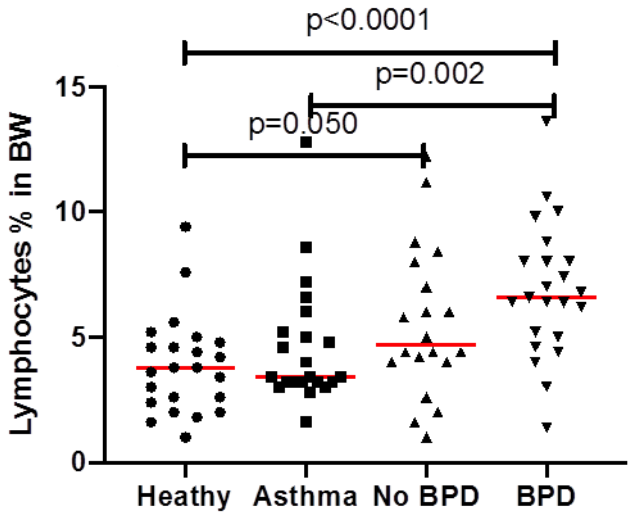

Figure 14. Proportion of lymphocytes in bronchial wash in healthy-, asthma-, preterm (non BPD) - and BPD groups (%).

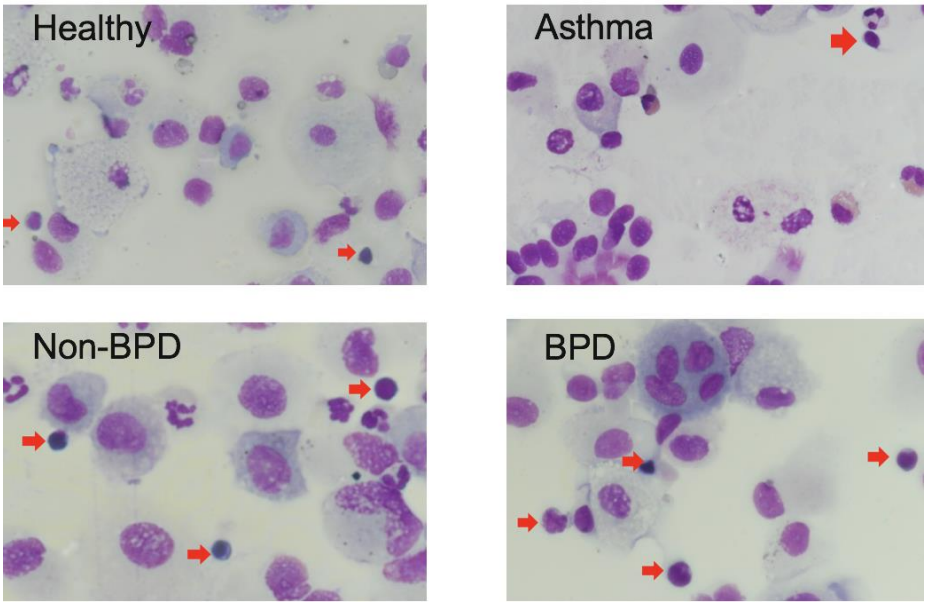

Figure 15. Micrographs of representative cytospins in healthy-, asthma-, preterm (non BPD) - and BPD groups. Red arrows mark the lymphocytes.

There was no sex difference in the distribution of eosinophils. However, the proportions of lymphocytes were different in males and females. The male subjects in the BPD group had more lymphocytes (7.4% [IQR 6.4-8.8]), when compared with non-BPD males (5.6% [IQR 4.2-6.3), male asthmatics (4.0% IQR 3.2-6.9) and healthy males (4.6 % [IQR 3.4-5.2]). In female subjects, differences in lymphocyte counts were only observed between BPD group and healthy group (5.8% [IQR 4.2-7.7] vs. 2.8% [IQR 2.0-3.8]).

Lymphocyte percentage was negatively associated with z-scores of post-bronchodilator FEV_1 and FVC only in male subjects born preterm, but not in females (Figure 16).

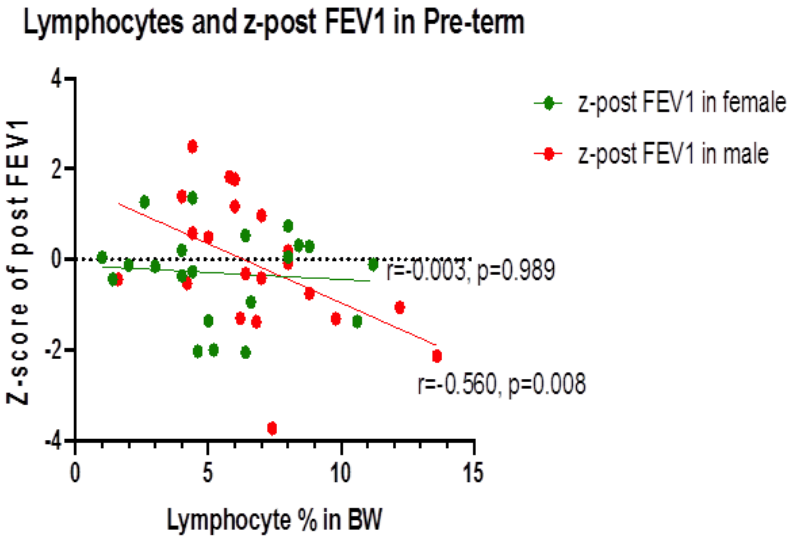

Figure 16. Correlation between lymphocyte proportions and FEV_1 in males and females born preterm.

6 DISCUSSION

6.1 STUDY COHORTS

The recruitment of participants to the studies was a challenge; the participating teenagers lived all over Stockholm and needed to take several days off from school, and the young adults had four separate visits to the hospitals finishing up with a bronchoscopy. Smokers had to be excluded as non-smoking was an inclusion criterion in order to allow the distinction of the impact on inflammatory cells due to preterm birth from that of the effect of smoking. The aim to recruit 24 subjects in each of the four study groups in LUNAPRE was only reached for the healthy control group. One of the unique features of the LUNAPRE study is the ability to make comparisons not only between groups of individuals born preterm with and without BPD and healthy controls, but also to a very well characterized control group of patients with mild allergic asthma. To explore the differences and/or similarities between the pulmonary consequences of preterm birth and asthma, a condition of obstructive airways disease that has been well investigated for decades was one of the central aims of the study. In both PALM II and LUNAPRE there were differences in GA and BW between the two preterm groups. However, we did not see any differences in the outcomes when adjusting for GA and BW in the studies. Further, we did not see any impact of SGA on the lung function parameters which has been reported by others.[254,255] My belief is that this is caused by the small sample size in our studies.

6.2 HEALTH RELATED QUALITY OF LIFE

The lack of differences in physical symptoms between the BPD group and healthy controls and more symptoms reported by asthmatics despite better lung function is to me personally not surprising but more confirmative and has also been demonstrated by Landry *et al*.[256] and Bozzetto *et al*.[257] Young adults with a history of BPD have probably had more or less airway obstruction since infancy[258] and are therefore likely to be adapted to this condition rather than the asthmatics who typically have acquired disease later during childhood or early adulthood and also might have a more variable disease. Being born preterm is associated with an increased risk of being diagnosed with developmental coordination disorder (DCD), attention deficit/ hyperactivity disorder (ADHD), attention deficit disorder (ADD), and autism spectrum disorder.[259-261] This might be reflected in that the mental component score was lower in both preterm groups than in the healthy control group.

6.3 LUNG FUNCTION AFTER PRETERM BIRTH

6.3.1 Measures of airflow limitation

In study I we demonstrated that preterm born adolescents with BPD had lower FEV_1 and the subjects with severe BPD also had lower FEV_1/FVC compared to preterm born adolescents without a history of BPD (non-BPD). Thirty-eight percent of the adolescents with BPD had FEV_1, and 33% had FEV_1/FVC below LLN. This in contrast with 0% FEV_1, and 20% FEV_1/FVC below LLN in the non-BPD group. Interestingly, we showed that these patterns remain in young adults with a history of BPD. In study II the BPD group demonstrated lower FEV_1, FVC and FEV_1/FVC, whereas the non-BPD group showed no differences compared to healthy controls. The asthmatics did differ in FEV_1/FVC ratio compared to healthy control and non-BPD groups but not to the BPD group. Nineteen percent of the young adults with BPD had post bronchodilator FEV_1, and 27% had post bronchodilator FEV_1/FVC below LLN. This in contrast to 0, 1 and 1% of the healthy controls the non-BPD group and the asthmatics, respectively.

We also investigated airflow limitation in the small airways using IOS and found in adolescents with severe BPD signs of peripheral airway obstruction. However, in adulthood there were no significant differences when comparing the BPD group to the non-BPD, asthma and healthy control groups. Notably, we only observed the IOS differences in the severe BPD group in adolescence, not in all BPD groups.

In study I we looked into lung development over time, from childhood to adolescence. We found a progress in airflow limitation measured by FEV_1/FVC and frequency dependence of resistance (R_{5-20}) in the severe BPD group. The persistent changes of airflow limitation in adults born preterm with a history of BPD are confirmative to other studies.[256,262]

Both preterm groups, in particular those with a history of BPD had a high incidence of airway hyper-responsiveness (AHR). AHR is a characteristic feature of asthma but also seen in patients with COPD. Some of the mechanisms behind AHR are considered to be airway inflammation, especially eosinophilic but also neutrophilic; and airway remodelling, including increased airway smooth muscles.[263,264] In a systematic review and meta-analysis Kotecha S *et al* reported an increased risk of AHR in children born preterm, especially in children with a history of BPD. It has been noted that in children born preterm AHR is not associated with eosinophilic inflammation.[265] Working capacity in children, adolescents

and adults born preterm has been evaluated using altering techniques in different studies and it is therefore difficult to compare the studies. Most studies find evidence of, or a trend towards, less work capacity in individuals born preterm with a history of BPD as we did in PALM II.[248,249]

6.3.2 Measures of lung volumes

We found no differences in lung volumes, measured as RV %pred. and TLC %pred. between the BPD and non-BPD groups in study I. In Study II the BPD group demonstrated reduced TLC %pred. compared to non-BPD-, asthma- and healthy control groups but RV %pred. and ratio RV/TLC showed no differences between the four groups. The alveolarization continues after birth and TLC plateaus at around 25 years of age. In study I we found a greater catch-up between school age and adolescence in FVC than FEV_1 in the BPD group compared to non-BPD group. This might implicate an enhanced lung growth regarding the volumes but not reflected in the airways which remain more obstructive.

6.3.3 Ventilation inhomogeneity

Ventilation inhomogeneity measured as LCI by MBW was increased in study II in the BPD group compared to non-BPD-, asthma, and healthy groups. This is considered a measure of small airway disease and the method is commonly used in patients with cystic fibrosis. Our finding is congruent with a study by Caskey *et al*.[262] Ventilation inhomogeneity can reflect areas in the lung with air-trapping due to narrowed small airways making it difficult to empty the inhaled air a feature also observed in obstructive lung diseases such as severe asthma and COPD. The asthmatics in LUNAPRE were diagnosed with mild disease and were therefore unlikely to show signs of air-trapping.

6.3.4 Diffusing capacity

In LUNAPRE a low gas diffusing capacity was observed in the BPD group, but this was also found in the preterm group who had normal expiratory flow volumes, confirming earlier findings.[262] Diffusing capacity is not only mirroring the function of the alveoli but also the capillaries surrounding them. Preterm birth per se is not only affecting lung development, it has been suggested that a decreased microvascular surface result in lower DL_{CO} also in individuals born preterm without BPD.[266]

6.3.5 FeNO, eosinophils in blood, BAL and bronchial wash

The asthma group was well-defined with all subjects sensitized to at least one airborne allergen. In the asthma group we found higher FeNO, blood eosinophils, BAL eosinophils

and BW eosinophils in contrast to the BPD group who did not differ from the healthy control group in this respect. This indicates an eosinophilic inflammation in the airways in the asthma group but not in the BPD group. The BPD group was to lesser extent sensitized to airborne allergens. This is of importance considering the choice of pharmacological treatments for the patients with BPD, and could imply that they are less likely to respond to conventional asthma treatment such as inhaled corticosteroids.

6.4 IMMUNE CELL PROFILES IN THE AIRWAYS AFTER PRETERM BIRTH

6.4.1 Bronchoalveolar lavage

LUNAPRE is to our knowledge the first study to investigate the mechanisms behind airway obstruction in adult individuals born preterm by bronchoscopy and BAL. We compared preterm subjects with an asthma patient group with airway obstruction where the inflammatory pattern is better known; and also to healthy never-smoking controls.
The T-cell profiles in young adults with a history of BPD demonstrated elevated cytotoxic T cells ($CD8^+$), decreases T-helper cells ($CD4^+$) and a lower ratio CD4/CD8 which are features seen in patients with COPD.[267] In the BPD group we also found indications that the cytotoxic T cells were activated due to a higher proportion of the early but unspecific marker CD 69. The correlation we found in the preterm groups between $CD8^+$ T cells, $CD4^+$ T cells and ratio CD4/CD8 and spirometry measures of airway obstruction may give insights about underlying disease mechanisms. The preterm born groups also demonstrated correlation between PD_{20}, spirometry reversibility testing and $CD8^+$ T cells and ratio CD4/CD8, respectively. It would be of great interest to measure markers like granzyme B and perforins to further confirm the action of $CD8^+$ T cells.

6.4.2 Bronchial wash

The pathophysiology of airways obstruction may be located in various parts of the airways. In order to mechanistically investigate involvement in the large airways we recruited cells by bronchial wash. The cell profile in the larger airway is unknown in individuals born preterm. Bronchial wash findings in both preterm groups with elevated lymphocytes indicate an involvement of both innate and adaptive immune system.[268] As with BAL T cells the intraepithelial lymphocytes in bronchial wash correlated with airway obstruction, particularly in males. To further investigate the lymphocyte subtypes would be of high interest. The sex differences may suggest that there are different mechanisms related to long-term effects of BPD in males vs. females.[269]

7 ETHICAL CONSIDERATIONS

Research in paediatric populations is the source of much ethical debate. There are many longitudinal studies that involve the collection of information as well as biological samples over extended periods of time. This data often spans over many years. Adolescents who are minor age are not independent decisions-makers and may not fully understand information of the research project. According to the standards for research in children, the parents or the legal guardian should represent the rights and the best for child in this situation. When the parent and child disagree (parent pro and child con), we always listen to the child´s opinion and do never impose any investigation without full cooperation. Furthermore, all collected research, data stored on computers and servers must adhere to high standards of security. In the PALM II and LUNAPRE cohorts we aligned the study protocols in order to adhere to high standards of ethical conduct. The participants were informed of their right to withdraw study participation at any time and without providing any motives for such decision. To promote data security all data referring to personal identity were coded in the database (which is stored on intra-net servers, protected by fire walls). Data will only be presented in aggregated level in publications. Furthermore, no individual data on study subjects is available to persons outside the study team and blood samples are stored in locked freezers and utilized according to standards set by Karolinska Institutet. There may come a time when the participants may no longer want their information to be available. In this case, individual level data will be expunged. In PALM II all parents and adolescents invited to participate received oral and written information of the procedures, and signed informed consent. In LUNAPRE the participants were given oral and written information and all participants signed consent at the inclusion. When completing participation in LUNAPRE the participants received 3000 SEK as compensation for time and inconvenience. This was approved by the KI ethical review board, and this type of incentive has been given in comparable Swedish studies. The long-term purpose with my research is to improve life-long health for survivors after preterm birth, today, and tomorrow. In the present studies, there has been little or no risk for harm, except for possible temporary inconvenience when collecting the data. Several participants are expected to benefit from the test results on an individual level, both in early detection of pulmonary problems of clinical significance at present, and in judging the need for future follow-up.

Consider that, the advantages (improved care) with the proposed study outweigh possible disadvantages (integrity concerns or inconvenience).

8 CONCLUSIONS

- Adolescents and young adults born very preterm with a history of BPD have expiratory flow values below the normal range and demonstrate hyper-responsive airways in adulthood.

- Adolescents and young adults born preterm without a history of BPD have expiratory flow values in the normal range but demonstrate hyper-responsive airways in adulthood.

- Young adults born preterm with and without a history of BPD have impaired gas diffusing capacity suggesting altered lung development of the immature lung.

- Young adults born preterm with a history of BPD report less symptoms than asthmatics despite lower lung function measures. Young adults born preterm with a history of BPD showed no signs of eosinophilic inflammation in the airways.

- Reduced proportion of T-helper cells ($CD4^+$), increased cytotoxic T cells ($CD8^+$) and reduced CD4/CD8 ratio in young adults with a history of BPD is compatible with the hypothesis that T-cells play a role in development of airway obstruction in this group of patients and suggest similarities to what is previously described in COPD

- Increased proportion of intraepithelial airway lymphocytes in adults with a history of BPD, particularly in males, indicate an involvement of both adaptive and innate immune response. The sex differences may suggest that there are different mechanisms related to long-term effects of BPD in males vs. females

- Overall, these studies suggest that objective measurements of lung function and long term follow-up of patients with BPD is of importance, and that pharmacological treatment needs to be tailored for this group of patients.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Mellan 6 och 12 % av alla barn föds för tidigt, det vill säga före 37 fullföljda graviditetsveckor. Överlevnaden efter för tidig födsel har ökat i hela världen efter att man för cirka 35 år sedan introducerade rutinen att ge kortison till gravida kvinnor som löper risk att föda för tidigt. Dessutom har den neonatala intensivvården utvecklats och i Sverige överlever nu åtta av tio barn som föds *extremt för tidigt*, det vill säga före 28 graviditetsveckor. Barn som föds *mycket för tidigt* (före 32 graviditetsveckor) löper ökad risk att drabbas av lungkomplikationer senare i livet. Detta beror dels på att lungorna mognar sent under graviditeten, dels på att det för tidigt födda barnets lungor utsätts för risker i form av höga luftvägs tryck från respiratorvård, syrgasbehandling och infektioner. Den diagnos som används för lungsjukdom på grund av för tidig födelse är bronkopulmonell dysplasi (BPD), vilket är ett vanligt resttillstånd efter extrem för tidig födelse. Då allt fler för tidigt födda barn idag överlever till vuxen ålder kommer sannolikt förekomsten av patienter med nedsatt lungfunktion på grund av BPD att öka framöver på vuxenmedicinska kliniker. Flera studier har visat att denna grupp har nedsatt lungfunktion redan i spädbarnsåldern och att de bär med sig funktionsnedsättningen under uppväxtåren upp till vuxen ålder. Många patienter uppvisar symptom som ses vid astma med en liknande bild vid lungfunktionsundersökning. Många patienter får också samma typ av behandling som patienter med astma, trots att det helt saknas behandlingsstudier. Ungefär 20-25% av alla vuxna patienter med kroniskt obstruktiv lungsjukdom (KOL) har aldrig rökt. I västvärlden minskar andelen dagligrökare, och är i Sverige nu under 10 %. Andra riskfaktorer än rökning för utveckling av KOL diskuteras nu, bland annat för tidig födsel

Syftet med denna avhandling var att studera kliniska, funktionella och mekanistiska aspekter på lungsjukdom hos för tidigt födda barn, både med och utan BPD, frånungdomsåren och upp till ung vuxen ålder.

Delarbetena i denna avhandling baserar sig på två studier, PALM II (ungdomar) och LUNAPRE (unga vuxna). Deltagarna i PALM II- och LUNAPRE- studierna bestod av individer som var mycket för tidigt födda med och utan BPD födda i Stockholm mellan 1992 och 1998. I LUNAPRE-studien ingick också lindrigt allergiska astmatiker och friska kontroller. Studiedeltagarna genomgick ett flertal lungfunktionsundersökningar och i PALM II gjordes även ett ansträngningstest. I LUNAPRE gjordes bronkoskopi där man med ett böjligt instrument direkt kan inspektera luftvägarna och ta prover, både från stora och små luftvägar. Även blodprover togs för att undersöka tecken till inflammation och

allergisk sensibilisering. Deltagarna besvarade dessutom flera frågeformulär om symptom, hälsa, livsstil och bakgrundsfaktorer. Information om nyföddhetsperioden och födelseuppgifter inhämtades från journaler och från medicinska födelseregistret.

Avhandlingen visar att ungdomar och unga vuxna med BPD hade luftflödesbegränsning till skillnad från de för tidigt födda utan BPD. Dock hade bägge grupperna en ökad känslighet i luftvägarna samt tecken till en nedsatt förmåga till gastransport mellan lunga och blod. Sammansättningen av vita blodkroppar från de små luftvägarna visade att de för tidigt födda med BPD hade en bild liknande den som ses hos vuxna patienter med KOL. BPD-gruppen hade alltså en lägre andel av så kallade T-hjälparceller och en högre andel av cytotoxiska T-celler och ett stört förhållandet dem emellan. Vid analys av prover från de stora luftvägarna fann vi att patienter med BPD, främst männen hade högre andel av en sorts vit blodkropp, lymfocyter. Vi såg också ett samband mellan dessa celler och luftflödesbegränsningen. Astmatikerna i studien hade flera tecken på inflammation av eosinofil typ vilket är karaktäristiskt vid allergisk astma. Så var inte fallet med för tidigt födda individerna.

För tidigt födda med BPD rapporterade mindre fysiska symptom jämfört med astmatikerna trots att de uppvisade mer luftflödesbegränsning. Både de för tidigt födda, med och utan BPD, rapporterade sämre psykiskt välbefinnande jämfört med den friska kontrollgruppen

Sammantaget visar denna avhandling att ungdomar och unga vuxna med BPD uppvisar en bild med luftflödesbegränsning och inflammatorisk cellbild i luftvägarna som liknar den som ses vid KOL. Trots detta sågs inte mer fysiska symptom än hos friska kontrollpersoner. Det är därför av stor vikt att följa för tidigt födda personer med BPD avseende lungfunktion till vuxen ålder och att studera orsakerna till luftflödesbegränsning för att i framtiden kunna välja lämplig behandling för dessa patienter.

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